Institut für Veterinärphysiologie

der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Max Gassmann

Arbeit unter wissenschaftlicher Betreuung von

Prof. Dr. med. vet. Thomas A. Lutz und Dr. Neil Docherty

Effect of Intensive Medical Management and Weight Loss on Urinary Biomarkers of Diabetic Kidney Disease in the Zucker Diabetic Sprague Dawley (ZDSD) Rat

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vorgelegt von

Michaela Hutter

Tierärztin

von Oberriet-Kriessern SG

genehmigt auf Antrag von

Prof. Dr. med. vet. Thomas A. Lutz, Referent

Dr. Neil Docherty, Korreferent

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Summary

Background:

Diabetic kidney disease (DKD) is one of the most common and potentially most serious complications of Type 2 Diabetes affecting up to 40% of patients. Recent clinical studies suggest a beneficial role for bariatric/metabolic surgery in the treatment of DKD, the most notably Roux-en-Y gastric bypass (RYGB) procedure. The role of surgery, as an adjunct to medical therapy or as a stand-alone treatment that enables anti-diabetic medication to be withdrawn, is a source of current debate in this context. In the present study we used the Zucker Diabetic Sprague Dawley (ZDSD) rat, as a pre-clinical model of DKD to compare the trajectory, of urinary biomarkers of renal injury in the ZDSD rat, before and at 4 week follow-up in different intervention groups; 1) Sham surgery 2) Sham surgery plus 15% body weight loss and intensive medical therapy (Best Medical Treatment (BMT) 3) RYGB surgery and 4) RYGB surgery plus intensive medical therapy (RYGB-BMT).

Method:

Weight and glycaemia matched ZDSD rats were assigned to either the Sham (n=9), BMT (n=7), RYGB (n=9) or RYGB-BMT (n=9) groups when 25 weeks old. The medical regimen included metformin (300 mg/kg), rosuvastatin (10 mg/kg), fenofibrate (100 mg/kg) and ramipril (1 mg/kg) for all medically managed rats and titrated Liraglutide only for the weight loss group. Dietary weight loss of 15% was achieved through food restriction. Weight and blood glucose was measured weekly. Before and 4 weeks post intervention initiation, rats were placed in metabolic cages for urine collection. After 17 hours urine samples were collected and analyzed by multiplex ELISA for albumin, neutrophil gelatinase-associated lipocalin (NGAL), osteopontin (OPN) and kidney injury molecule 1 (KIM-1). Age and sex-match Sprague Dawley rats served as healthy controls in the study.

Results:

Urinary excretion of albumin ($r = 0.47$), NGAL ($r = 0.71$), OPN ($r = 0.64$) and KIM-1 ($r = 0.55$) correlates with the degree of hyperglycaemia ($p < 0.01$, Pearson's test). Urinary albumin excretion is reduced in rats following BMT and RYGB (BMT) ($p < 0.05$, Wilcoxon test). In parallel, NGAL excretion into the urine increased in both BMT and BMT-RYGB groups ($p< 0.05$, Wilcoxon test). An elevation of the OPN excretion rate could be observed in the BMT and RYGB group ($p< 0.05$, Wilcoxon test), while none of the interventions had an influence on KIM-1 excretion rate.

Conclusion:

Intensive weight loss (dietary or arising post-surgery) in combination with intensive medical therapy effectively reduces hyperglycaemia and urinary albumin excretion in

ZDSD rats. The outcomes are better than in animals only undergoing RYGB surgery and without medical treatment. However, persistent elevation of urinary NGAL is suggestive of ongoing of tubular injury, potentially attributable to an aspect of medical treatment, rate of weight loss or a combination of both.

Zusammenfassung

Hintergrund

Die diabetische Nephropathie (DKD) ist eine der häufigsten und wahrscheinlich die gefährlichste Komplikation von Diabetes Typ 2. Über 40 % der Patienten können betroffen sein. Neuere Studien zeigen einen positive Effekt von bariatrischen/metabolischen Operationen als Therapie von DKD, besonders die Roux-en Magenbypass Operation (RYGB). Die Rolle der Operation in Kombination mit Medikamenten oder als eigenständige Therapieform, die das Absetzen der anti-diabetischen Therapie ermöglicht, wird zurzeit heftig diskutiert. In dieser Studie benutzen wir Zucker Diabetic Sprague Dawley (ZDSD) Ratten als präklinisches Model für DKD. Die Tiere wurden in folgende Gruppen eingeteilt1) Sham Operation 2) Sham Operation plus 15% Körpergewichtsverlust und medikamentöse Therapie (Best Medical Treatment (BMT) 3) RYGB Operation und 4) RYGB Operation plus medikamentöse Therapie (RYGB-BMT). Anschliessend wurden diverse Biomarker für Nierenschaden bevor und vier Wochen nach der Intervention gemessen.

Methode:

Die ZDSD Ratten wurden nach Gewicht und Glykämie normalisiert und dann entweder der Sham (n=9), BMT (n=7), RYGB (n=9) oder RYGB(BMT) (n=9) Gruppe zugeordnet, als sie 25 Wochen alt waren. Das Medikamentenregieme bestand aus Metformin (300 mg/kg), Rosuvastatin (10 mg/kg), Fenofibrate (100 mg/kg) und Ramipril (1 mg/kg) für alle Tiere in den medikamentösen Gruppen und zusätzlich Liraglutide nur für die BMT's. Ein 15 %-iger Gewichtsverlust wurde durch Futterreduktion herbeigeführt. Das Gewicht und der Blutzucker wurden wöchentlich gemessen. Bevor und 4 Wochen nach dem Beginn der Interventionen, wurden die Ratten für 17 Stunden in metabolischen Käfigen gehalten. Anschliessend wurde der Urin eingesammelt und via multiplex ELISA auf Albumin, Neutropile gelatinase-associated lipocalin (NGAL), Osteopontin (OPN) und Kidney injury molecule 1 (KIM-1) untersucht. Sprague Dawley Ratten desselben Geschlechts und Alter galten als Kontrollgruppe.

Resultat:

Die Urinausscheidung von Albumin ($r = 0.47$), NGAL ($r = 0.71$), OPN ($r = 0.64$) und KIM-1 ($r = 0.55$) korrelierte mit der Hyperglykämie ($p < 0.01$, Pearson's Test). Die Albuminausscheidung war reduziert nach BMT und RYGB (BMT) ($p < 0.05$, Wilcoxon Test). Parallel dazu, erhöhte sich die NGAL-Ausscheidung in beiden Gruppen ($p< 0.05$, Wilcoxon Test). Erhöhte OPN-Ausscheidung konnte in der BMT und RYGB Gruppe beobachtet werden ($p< 0.05$, Wilcoxon Test). Keine der Interventionen hatte einen Einfluss auf die KIM-1 Ausscheidung.

Konklusion

Intensiver Gewichtsverlust (diätetisch oder nach einer Operation) in Kombination mit intensiver medikamentöser Therapie reduziert die Hyperglykämie und Albuminausscheidung in ZDSD Ratten beträchtlich. Die Resultate waren besser, als in den Tieren, die nur eine RYGB Operation ohne medikamentöse Therapie hatten. Doch die erhöhten NGAL Werte deuten auf einen fortlaufenden tubulären Schaden hin. Möglicherweise verursacht durch die medikamentöse Therapie oder massiven Gewichtsverlust, oder einer Kombination von beidem.

1 Introduction

1.1 Diabetic kidney disease in humans

1.1.1 Epidemiology of diabetic kidney disease

Worldwide over 420 million people have diabetes. The prevalence was 5.6% in Switzerland in 2016(1). About 30 % of them will develop diabetic kidney disease (DKD) and a small amount will progress to end stage renal disease (ESRD) (2)

1.1.2 Symptoms of diabetic kidney disease

The typical patient with DKD has a long history of diabetes, retinopathy and albuminuria, but without hematuria (3). Retinopathy can be absent in type 2 diabetes mellitus (T2DM) patients. DKD can be already present at diagnosis of T2DM. In type 1 diabetes mellitus (T1DM) patients, DKD usually evolves 10 years after the diagnosis (4). Albuminuria can also be absent in DKD patients. Recent reports have shown the absence of albuminuria in some patients with reduced glomerular filtration rate (GFR)(5, 6). Sixty to 70% of the T2DM patients, who do not have proteinuria, have hypertension. Hypertension can cause development or progression of DKD. When the kidney is damaged, different factors, like the activation of the renin-angiotensin-aldosterone-system (RAAS), can increase hypertension, thus creating a vicious circle. Mortality in patients with DKD is more often attributable to fatal cardiovascular events which occur prior to transition to DKD.

1.1.3 Diagnosing diabetic kidney disease

Kidney damage is associated with albuminuria, elevated plasma creatinine and reduced GFR.

Albuminuria is mainly diagnosed in two ways, either by the albumin/creatinine ratio in the urine (UACR) in a random spot collection or via albumin measurement in a 24 hour urine sample collection (7). The 24 h urine albumin concentration test is the gold standard. However, the UACR is more convenient, because it can be performed during a general consultation. A UACR < 30 mg/g creatinine is considered normal, while n UACR ≥ 30 mg/g creatinine is increased. The sensitivity is 86 % and the specificity 60 % when UACR ≥ 30 - 299 mg/g and 75 % respectively 99 % when UACR ≥ 300 mg/g (8). Because the urinary albumin concentration is influenced by hydration, albumin should not be measured on its own (3). Fever, exercise, menstruation, hypertension and other factors can elevate UACR independent from kidney damage. The American Diabetes Association (2018) suggests repeating the UACR measurement within three to six months before diagnosing someone with albuminuria. In order to stage kidney disease, the estimated GFR (eGFR) should be calculated. At an early stage of CKD the eGFR increases due to the hyper filtration. Values of ≥ 120 ml/min/1.73 m^2 can be measured. From stage 3 on the eGFR will drop to < 60 ml/min/1.73 m^2 (9). More recently other kidney injury biomarkers have been proposed for use in prognostication in DKD and will be discussed in chapter 1.7.

1.1.4 Staging of chronic kidney disease

In 2014 the Joint Committee on Diabetic Nephropathy made a new classification for diabetic kidney disease (10):

Table 1

Classification	Urinary albumin [mg/g Cr] Urinary protein [g/g Cr]	eGFR [ml/min/1.73 m^2]
Stage I (prenephropathy)	normoalbuminuria < 30	≥ 90
Stage II (incipient nephropathy)	microalbuminuria 30-299	≥ 60-89
Stage III (overt nephropathy)	macroalbuminuria ≥ 300 or proteinuria ≥ 0.5	30-59
Stage IV (kidney failure)	albuminuria or proteinuria independent, no dialysis required	<30
Stage V (dialysis)	albuminuria or proteinuria independent, dialysis required,	< 30

1.1.5 Risk factors

Obesity and hypertension are important additional risk factors for developing CKD alongside diabetes (11). Treatment with antihypertensive medications such as angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) is suggested, if blood pressure rises above 140/90 mmHg. The objective is to reduce the risk of cardiovascular events, such as myocardial infarctions and stroke (12, 13). Patients with T2DM often show dyslipidaemia characterized by hypertriglyceridaemia, increased high-density lipoprotein (HDL) and decreased high-density lipoprotein (HDL). Dyslipidaemia is associated with increased mortality and cardiovascular disease (CVD). Trough different pathomechanism high triglycerides and LDL can cause glomerular injury and increased matrix deposition into the tubulointerstitial space(14).

1.2 Pathophysiology of DKD

1.2.1 Histological findings DKD

In humans` glomerular basement thickening, mesangial expansion and local to global glomerulosclerosis are common findings in DKD. The glomerular changes can be classified from I-IV. In addition tubular basement membrane thickening, tubulointerstitial fibrosis and tubular atrophy occur. Infiltration of the interstitium by immune cells can be present (15). The glomerular classifications and especially the amount of interstitial fibrosis and tubular atrophy have most likely an impact on progression to ESRD (16). Podocyte damage, loss and modification play an important role in developing albuminuria. Foot process effacement hypertrophy, detachment from the basement membrane and decreased density are already present in an early stage of DKD (17). Insulin is important for podocytes to react to increased intraglomerular pressure and increased GFR. Podocytes secret vascular endothelial growth factor A

(VEGF-A), which affects the endothelium cells. Too much VEGF-A (most likely present in DKD) and not enough VEGF-A (late state DKD) has negative effects on endothelium cells, thus weakening the glomerular filtration barrier further and worsening albuminuria (18)

1.3 Pathophysiology DKD

Diabetic kidney disease has multiple risk factors including hypertension, hyperglycemia and inflammation. These risk factors interact with each other and enhance the progression of DKD.

1.3.1 Glucotoxicity

Hyperglycemia induces the production of superoxide products which inhibit glyceraldehyde 3-phosphate dehydrogenase (GADPH), which transforms glyceraldehyde-3 phosphate with the help of nicotinamide adenine dinucleotide (NAD^+) into 1,2 diphosphoglycerate. Glycolysis in the cell is reduced and more products upstream of 1,3 Diphosphoglycerate are available. This leads to activation of parallel substrate utilisation pathways. (19) The now increased glucose levels in the cell can lead to an augmented sorbitol and fructose production, by activating the polyol pathway. Increased fructose levels have been associated with proteinuria, and increased mesangial matrix expansion and tubular injury (20). In addition the production of sorbitol uses nicotinamide adenine dinucleotide phosphate (NADPH), leading to a decreased regeneration of glutathione, more reactive oxygen species (ROS) are formed (21). In summary increases production of sorbitol and fructose might lead, via ROS, to direct and indirect kidney damage.

More fructose-6-phospate leads to increased glucosamine 6-phospate (glucosamine-6P) production, via the hexosamine pathway. Glucosamine-6-P influences the production of tumour necrosis factor alpha (TNF-α), promoting inflammation. In addition it increases tissue growth factor beta(TGF-β), which leads to renal cell hypertrophy and augmented mesangial matrix deposition, narrowing the lumen of the glomeruli.(19) Glucosamine-6-P can be metabolized further and impair nitric oxide (NO) production, (22). High intracellular glucose levels enhance the production of advanced glycation products (AGE). They can impair and modify not only intracellular, but also extracellular proteins or increase the production (19). AGE's are associated with more extracellular matrix (ECM) accumulation in the glomeruli. Changes in collagen IV and laminin by AGE's have been demonstrated to increase the permeability of the glomerular basement membrane (GBM). AGE can bind to inflammatory receptors and enhance inflammation as well as increasing pro-fibrotic factors, like VEGF and connective tissue growth factor (CTGF), both associated with hypertension and proteinuria (19, 23, 24). In summary AGE's induce ECM expansion, increase GBM permeability, proteinuria and fibrosis.

Another important pathway is the protein kinase C pathway (PKC). PKC increases CTGF, TGF-β, collagen IV production enhancing further GBM thickening, glomerular and tubular hypertrophy and sclerosis(19). In addition PKC increases the activity of nitric oxide (NO), which dilates the afferent arteriole in the glomerulus. It also enhances the

vasoconstrictive effect of angiotensin II (Ang II) on the efferent arteriole, leading to glomerular hypertension and increased GFR (25).

1.3.2 Glomerular hypertension

Increased glucose levels in combination with hyperinsulinemia lead to augmented glucose reabsorption in the kidney. As a side effect sodium reabsorption in the proximal tubules is increased. As consequence the macula densa will sense low sodium levels and activate sodium retention mechanism, like the renin-angiotensin-aldosterone system RAAS). The volume expansion leads to dilatation of the afferent arteriole in the glomerulus. The intraglomerular pressure rises and the GFR increases.

Early stage of DKD is now present with an increased GFR. In addition renin, aldosterone and especially angiotensin have further effects on the kidney. Angiotensin increases the intra glomerular pressure by contraction of the efferent arteriole and can damage podocytes directly. The full RAAS enhances over different pathways kidney inflammation, renal cell loss, extracellular matrix accumulation, oxidative stress, mesangial cell expansion and glomerular hypertrophy, leading finally to DKD and progressing the disease(26).

Other factors are increased in patients with DKD. Endothelin 1 (ET-1), secreted by mesangial, glomerular and renal tubular cells, induces vasoconstriction, fibrosis, podocyte injury, mesangial cell proliferation and ECM production. It also activates receptors that can directly increase the glomerular permeability (27). Finally the amount of NO seems to decrease, reducing the vasodilatation of the afferent arteriole and more and more cells being damaged, leading to a reduced GFR and progression of DKD to ESRD (28).

1.3.3 Inflammation

Many inflammation factors, like TNF-α, interleukin-1 and interleukin-6 are increased in kidney of diabetic people. They can lead to apoptosis of endothelial cells, GBM thickening and increase of the permeability for proteins (19). Further inflammatory pathways have been found activated, like the Janus kinase/signal transducer and activator of transcription proteins (Jak/STAT) or the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Both pathways induce glomerular changes, leading to hypertrophy of mesangial cells, increasing GFR, apoptosis of other renal cells and proteinuria (29, 30).

In summary, there are many factors increasing ECM deposition, renal cell hypertrophy, apoptosis fibrosis, glomerular hypertension and inflammation. It leads first to an increased GFR and while the glomerulus becomes more and more crowded, the GFR decreases. The apoptosis of cells and change of the basal membrane leads to leakage of the glomeruli, becoming apparent by the proteinuria.

1.4 Therapy of diabetic kidney disease

1.4.1 Management of diabetic kidney disease patients

The American Diabetes Association (ADA) suggests starting DKD treatment before the patients show albuminuria. The goal is to prevent the development of albuminuria by controlling risk factors such as hyperglycemia, hypertension, dyslipidaemia and smoking (3). In addition the patient's urinary albumin should be revaluated at least once a year.

If microalbuminuria is present, it is the goal to prevent progression to macroalbuminuria. Hyperglycaemic, hypertensive, dyslipidaemic control have to be intensified. Good glycemic control can reduce the progression to ESRD (31). Dietary changes (reduced protein and salt intake) and moderate exercise should be considered. When the GFR is depressed, drug dosage adjustment should be made. Vaccination against Hepatitis B should be considered and bone density and vitamin D supplementation controlled. If the GFR further declines electrolytes, bicarbonate, calcium, phosphorus, parathyroid hormone, haemoglobin, albumin, and weight should be controlled every 3–6 months. When the GFR < 30 ml/min/1.73 m2 the patient should be referred to a nephrologist to discuss renal replacement therapy (32).

1.4.2 Dietary suggestions

1.4.2.1 Reduce protein

Protein intake in non dialysis dependant patients should be reduced to maximal 0.8 g/kg per day. A daily intake of more than 20 % protein, increases albuminuria and enhances the progression of CKD. However, the protein amount may have to be increased in dialysis dependant patients, due to proteinuria (33).

1.4.2.2 Reduce sodium and potassium

Patients with DKD often have salt sensitive hypertension and hyperkalemia. The reduction of sodium and potassium can lower blood pressure, protect excitable tissue function and have a positive effect on the progression of CKD (34, 35).

1.4.3 Glycemic control

Good glycemic control has been shown to decrease progression of DKD to ESRD (31). At an early stage patients with T2DM can be treated with metformin, but it is contraindicated if GFR < 30 ml/min/1.73 m^2 (33). If glycemic control cannot be achieved with only metformin, the American Diabetes Association (2018) suggests adding another drug, like GLP-1 receptor agonists or baseline insulin. The patient should be revaluated 3 months later. In case the glycemic target is still not reached, a triple drug therapy can be considered (36).

1.4.4 Antihypertensive therapy: Angiotensin-converting-enzyme (ACE) inhibitors /Angiotensin II receptor blockers (ARB's)

Hypertension is a risk factor for CKD. Blood pressure control is suggested from 140/90 mmHg on (12). Reduction of blood pressure decreases albuminuria significantly and reduces the risk of cardiovascular events (13). Both, ACE inhibitors and ARB slow down

the progression of CKD (37), but they should not be given in combination. Together they increase the risk of hyperkalemia and acute renal insufficiency (AKI) (38).
Antihypertensive therapy should not be started as long as the patients do not suffer hypertension, because otherwise the risk of cardiovascular events is too high (39).

1.4.5 Control of dyslipidaemia

Dyslipidaemia is associated with increased mortality and more prevalent in patients with DKD(14). Rosuvastatin blocks the production of LDL and increases the HDL in the blood. It reduces tubular damage in patients with DKD (40).
Fibrates are peroxisome proliferator-activated receptor (PPAR's) analoga. They improve the insulin sensitivity and the glucose uptake into the striate muscles and the adipose tissue. In a diabetic mouse model, fenofibrate reduced albuminuria and glomerular injury (41, 42).

1.4.6 Exercise/weight loss

Weight loss, especially in prediabetic patients has been shown to reduce the risk to develop diabetes significantly (43).

1.5 RYGB

1.5.1 What is RYGB?

Roux-en Y gastric bypass surgery (RYGB) can be used for weight loss and or as a treatment of type 2 diabetes. The stomach is divided into a smaller upper pouch (about 15 ml volume) and a bigger lower pouch (about 400 ml). The jejunum is transsected proximally. The distal end of the jejunum is then attached to the smaller upper stomach pouch to form an anastomosis to bypass most of the stomach and the duodenum. The remaining jejunum stump, continuous with the larger stomach remnant and the duodenum is then anastomosed to the downstream jejunum to form the Y shape of the reconstruction (44). The anastomosis could also be done in the ileum, but there are more complications. Because more of the small intestine is bypassed, there is more malabsorption (mainly starch and fat), reduced vitamin and mineral uptake and more undigested food arrives in the colon where micro flora can use it to produce gas(45, 46). The complication rate of the surgery is 4% within the first 90 days but up to 20% over the lifetime of the patient. Complications after surgery are: Prolonged nausea and vomiting, haemorrhages, wound infection and peritonitis, anastomotic leakage or stricture (44, 47).

1.5.2 Indications

At the 2nd diabetes surgery summit (DSS-II) the new guidelines for metabolic treatment were presented. For all obese people with a BMI ≥ 40 kg/m^2 and Asians ≥ 37.5 kg/m^2, as well as T2DM patients with poor glycemic control and BMI ≥ 35 – 39.9 kg/m^2 (Asians ≥ 32.5 -37.5 kg/m^2), bariatric surgery is recommended. For everyone with T2DM, good glycemic control and a BMI ≥ 35 – 39.9 kg/m^2 (Asians ≥ 32.5 -37.5 kg/m^2) or T2DM patients with bad glycemic control and a BMI ≥ 30 – 34.9 kg/m^2 (Asians ≥ 27.5 -32.4 kg/m^2) metabolic surgery should be considered as an alternative to medical treatment.

Bariatric surgery should not be performed in non obese or T1DM patients (32).

1.5.3 Advantages

The most important effect is the weight loss. In a Swedish obese study patients lost 25% weight over 20 years (48). Hyperlipidemia can be reduces in most patients. Hypertension is decreased in almost all patients. Type 2 diabetes improves in many of the patients, and the risk to develop T2DM can be reduced 30x in patients undergoing surgery in a prediabetic state (49). Bariatric surgery reduced the progression from early stage CKD to ESRD (50). In other studies the plasma OPN levels were monitored before and after bariatric surgery. Plasma OPN levels were high prior to surgery and did not decrease after surgery. This was possibly because of the increased bone markers, caused by the adaption of the bones to the weight loss (51, 52).

1.5.4 Risk factors

The disadvantages of bariatric surgery are the reduced uptake of vitamins, especially Vitamin B12 and fat soluble vitamins. These vitamins have to be taken as supplements (53, 54). The iron and calcium uptake is reduced, because the duodenum is bypassed, where most of it is absorbed. The low iron levels can lead to pica(55). There are reports of people suffering depression after surgery, because of the change of what can be eaten and the amount of it (56). Muscular weakness can occur, because of a reduced protein intake. It can lead to balance problem and increased fatigue end early exhaustion (54).Further complications of RYGB surgery are: Increased risk of gallstone formation and kidney stone formation (57, 58)

Bariatric surgery slightly increases the bone fracture risk, especially in combination with malabsorbtion (59, 60). Different mechanisms are proposed to be the cause. Malabsorbtion of Vitamin D leading to secondary hyperparathyroidism and reduced skeletal loading, because of weight loss, are associated with decreased bone density. Bone turnover markers are elevated (61, 62). In a previous study, Riedl *et al*. (2008) found that plasma OPN did not decrease after bariatric surgery. He explains it with the increased bone turn over, since OPN is involved in bone remodelling (63).

1.6 Rodent models for diabetes

There are many different rodent models for diabetes. In general, diabetes can be induced via injection of a chemical agent, such as Streptozotocin (STZ), provoked via diet, genetic defects or viral infections (64).

1.6.1 Type 1 diabetes mellitus models

Streptozotocin (STZ) can be used to induce diabetes in rats and mice (65). It destroys the β-cells in the pancreas. This model is often used for studying T1DM. It is cheap, but STZ can be toxic to other organs (66). Multiple injections of lower dose STZ leads to diabetes and it has less negative effects on other organs (65).

The non obese diabetic (NOD) mice develop diabetes at 12 – 14 weeks of age in females. T1DM is caused, due to severe pancreatic islet inflammation. In addition to diabetes,

NOD mice also are likely to develop other autoimmune diseases (67). There are other rodent models that will develop T1DM spontaneously, like the BB rat.

AKITA mouse has a mutation of the Ins2 gene resulting in abnormal folding of Insulin, which then loses functionality. These animals only exhibit modest kidney changes and are not an ideal model of T2DM. (68)

There is the possibility to induce diabetes via viruses, but this is more appropriate to understand the involvement of viruses in the pathogenesis of T1DM (64).

1.6.2 Type 2 Diabetes rodent models

1.6.2.1 Mice

1.6.2.1.1 Leptin receptor deficient mice

Leptin-deficient ob/ob and db/db mice have a defect in the leptin receptor. This leads to hyperphagia, obesity, hyperinsulinemia, hyperlipidemia and finally to insulin resistance. Kidney changes can be observed, like reduced GFR, ECM expansion and podocyte loss. Tubulointerstitial fibrosis and nodular sclerosis does not occur and therefore does not exactly mimic the changes observed in humans. Leptin deficiency is also a very rare cause for T2DM in humans (69).

1.6.2.1.2 Diet induced diabetes

Mice, usually from the C57BL/6 strain, can be fed a high fat diet to develop obesity, insulin resistance, dyslipidaemia and hyperglycaemia. Environmental conditions and gender also play important roles (70).

1.6.2.2 Rats

1.6.2.2.1 Zucker Diabetes Sprague Dawley (ZDSD) rat

The ZDSD rat is bread from a lean Zucker Diabetic Fatty (ZDF) male rat and a female member of the Crl:CD(SD) rat and then selectively bred for obesity. They have an intact leptin pathway. Male ZDSD develop spontaneously T2DM with normal chow (71). ZDSD have a long prediabetic state, starting already at seven weeks of age. They show signs of metabolic syndrome with hyperglycemia, hyperinsulinemia, decreasing glucose tolerance and increasing insulin resistance and hyperlipidemia. The long prediabetic period mimics what happens to humans who develop T2DM. They start developing overt diabetes, when 19 weeks of age. Insulin decreases, because of β- cell failure. They exhibit polyuria and polydipsia and glucose intolerance (71). When they are 24 weeks old they are likely to develop complications as diabetic kidney disease with progressive albuminuria and decreased GFR. Urinary kidney injury markers such as kidney injury molecule 1 (KIM-1), β-microglobulin, clusterin and cystatin are elevated. ZDSD rats also develop glomerular basement membrane (GBM) thickening and mesangial matrix expansion, making it to a better model for DKD than most other rodents. However, ZDSD are a rather new model and a lot of information about the pathogenesis of DKD in this rats is still missing (72).

1.6.2.2.2 Zucker Diabetic Fatty (ZDF) rat and other leptin receptor deficient rats

The ZDF rats have a defect in the leptin receptor and also a defect in the β-cell transcription (73). It is one of the most used rodent models for T2DM. Male homozygous fa/fa ZDF develop obesity spontaneously, because of hyperphagia. When 8 weeks old the β-cells start failing, and T2DM develops fast, without a long prediabetic state. Kidney changes are moderate, only at a late stage (at 22 weeks) they develop focal and segmental tubulosclerosis and moderate mesangial expansion (74). In addition they can develop hydronephrosis, which does not make them ideal to study DKD. In addition leptin receptor defect is a rare cause for T2DM in humans (69).

The ZSF-1 rat were bred from ZDF and a spontaneously hypertensive heart failure (SHHF) rat. Like the ZDF they have a mutation in the leptin receptor. They become obese, due to hyperphagia and the males will develop T2DM with hyperglycemia and proteinuria. Their kidney changes are more similar to the human DKD form. They show tubulointerstitial fibrosis, mesangial expansion and (GBM) thickening and tubules dilatation and atrophy (75, 76). They also show a severe hyperlipidemia, which is not present in this form in humans with T2DM (76).

1.7 Urinary kidney injury markers

The following urinary kidney injury markers were used in this study to assess kidney injury in the used rodent models: neutrophile-gelatinase-associated lipocalin (NGAL), Osteopontin (OPN), kidney injury molecule 1 (KIM-1)

1.7.1 Neutrophil gelatinase- assosciated lipocalin (NGAL)

1.7.1.1 Pathophysiology of NGAL

NGAL is also known as lipocalin 2. It is from the lipocalin superfamily. The protein consists of eight antiparallel β-sheets forming a cup and 2 α helices (77). It is expressed and secreted by different cell in the body including hepatocytes and renal tubular cells, when pathology present. NGAL has a bacteriostatic effect by capturing and depleting siderophores, thus reducing the free iron (77). It is involved in the growth and differentiation of mature renal epithelium and its organisation in the tubules.

1.7.1.2 NGAL obesity

NGAL expression is up regulated in adipose tissue of obese mice and humans (78). De Muro et al (2016) showed that urinary NGAL (uNGAL) can be elevated before the onset of microalbuminuria. He highlights that uNGAL is increased when tubulus damage occurs, which one of the early changes in the diabetic kidney(79).

1.7.1.3 NGAL and the kidney

NGAL is a good marker for tubulo-interstitial damage. In case of kidney damage it is mainly up regulated in the ascending loop of Henle, the distal tubules and the collecting duct (77). NGAL is up regulated after ischaemic or nephrotoxic acute kidney injury, but it can be also up regulated in chronic kidney disease and is an important factor for progression, even independent from the GFR. Its expression in the kidney correlates with the severity of the lesions (80, 81). In a study with 77 patients with T2DM high a level of urinary NGAL excretion was associated with a faster decline of the GFR and progression of DKD (82)

1.7.2 Osteopontin

1.7.2.1 Pathophysiology of osteopontin

Osteopontin (OPN) was first discovered in 1985 (83) and is also known as secreted phosphoprotein 1 (SPP) or early T-lymphocyte activation (ETA-1). It is a small integrin-binding ligand, N-linked glycoprotein (SIBLING) protein (84). OPN is encoded by the *SPP 1* gene, it has 7 exons, is 5kb long and can be found on chromosome 4 in humans (85). The *SPP 1* gene in mice is located on chromosome 5 (86) and in the *Rattus norvegicus* on chromosome 14. The size of OPN differs from 41 – 75 kDA, because of post-translational modifications (87). OPN can be found in all body fluid, such as milk, urine, blood liquor. It is secreted in many locations, including cartilage, teeth, kidney: especially podocytes and in the distal renal tubules, brain, vascular tissues, mammary, activated macrophages and lymphocytes (88). OPN is secreted as full length OPN, OPN-b lacks exon 5 and OPN-c lacks exon 4 (89). There are signs that different forms of OPN might be present at different stages of diseases. Kiefer (2008) found that OPN-a was reduced in the adipose tissue of obese people, while OPN – b was elevated and OPN – c unaltered.

OPN exists in different forms. The secreted form (sOPN) works as a cytokine and its intracellular form (iOPN), found in the cytoplasm, is responsible for the arrangement of the cytoskeleton. Both forms are generated from the same DNA sequence, but are then differentiated through different translational initiation sites (90). sOPN is involved in the immune response regulation, by decreasing IL-12 and increasing IL 10 (91). It can inhibit apoptosis via integrins (92) and OPN seems to play an important role in tumour progression and metastasis (93, 94). OPN affects most cells via different αβ-Integrins, that are mainly binding to the Arg-Gly-Asp (RGD)- binding site of OPN (87). It can also bind to CD44 (Asou 2001).

OPN plays a role in bone remodelling and bone formation and it stimulates the adhesion, migration and bone resorption by osteoclasts (95). Elevated levels of OPN have been found in autoimmune diseases such as multiple sclerosis or lupus erythematosus (96, 97). OPN is elevated and involved in the mineralization of soft tissue for example vascular calcification and valvular calcification (98, 99). It also plays a role in the formation of renal crystals (100). OPN can modulate the inflammatory response by modulating the migration and cytokine release of white blood cells (101).

1.7.2.2 OPN in obesity and diabetes

Elevated OPN has been found in the adipose tissue of obese patients. Macrophages are thought to be the main source of the OPN; during adipose tissue inflammation, interestingly plasma OPN was just slightly increased (102). In OPN $^{-/-}$ obese mice, the amount of macrophages in the adipose tissue was decreased, suggesting that OPN plays also a role in the attraction of macrophages (103). Dietary weight loss and exercise reduces the OPN level (104). Kahles (2014) suggests that OPN plays a key role in the development of insulin resistance when rats are fed with high fat diet. The reason is the impairment of the differentiation and insulin sensitivity of adipocytes, mediated by OPN through different receptors (105).

1.7.2.3 OPN and CKD

OPN is expressed by the distal tubular epithelial cells. In the case of kidney damage, it can also be up-regulated in the glomeruli and by the macrophages found in the parenchyma (106, 107). Increased plasma OPN was associated independently of the severity of the nephropathy and albuminuria in patients with T2DM (108). OPN -/- mice did not have albuminuria and were protected from renal damage (109). OPN is responsible for the stress adaption in podocytes, caused by hypertension (Schordan 2010). The administration of ACE inhibitors reduces the OPN level (106).

1.7.3 Kidney Injury molecule 1 (KIM-1)

1.7.3.1 Pathophysiology of Kim-1

Kidney injury molecule 1 (KIM-1) is also known as T-cell immunoglobulin domain and mucin domain 1 (TIM-1) or hepatitis A virus cellular receptor 1 (HAVcr-1). It is a type 1 membrane glycoprotein with 6 cysteine immunoglobulin-like domains and a mucin domain and N- and O-glycosylation sites. In the kidney, it is involved in the apoptotic cell phagocytosis and acts as a receptor to transform the proximal tubulus epithelium cells into semi-professional phagocytes enhancing their uptake of necrotic cell debris (110).

1.7.3.2 KIM-1 and chronic kidney disease

KIM-1 is not detected in healthy kidneys, but it is highly up regulated in the proximal tubules epithelium in case of acute or chronic kidney injury. A huge amount of it is secreted into the urine, making it a good biomarker for kidney damage (111).

1.7.3.3 KIM 1 and diabetes

Increased KIM-1 levels in the urine have been described in patients with type 1 and type 2 diabetes mellitus. Vaidya et al. (2011) reported that a regression of albuminuria led to a decrease of KIM-1 in patients with T1DM (112). Patients with an increased KIM-1 without proteinuria progress earlier to more severe kidney diseases stages (113). Nielson et al. (2012) described a faster decline of the GFR in T2DM patients with elevated KIM-1 in the urine (82)

KIM-1 is elevated in obese patients with normal kidney function. Six months after RYGB surgery the urinary KIM-1 levels increased and decreased 1 year post surgery. (114)

1.8 Research Questions

The overarching research question under investigation in this thesis was to explore the differential impact of types and degrees of weight loss with and without intensive medical therapy on excretion of urinary biomarker surrogates of kidney injury. I hypothesised that ZDSD rats after RYGB combined with best medical treatment would have lower urinary kidney injury marker levels. The aims of my study are:

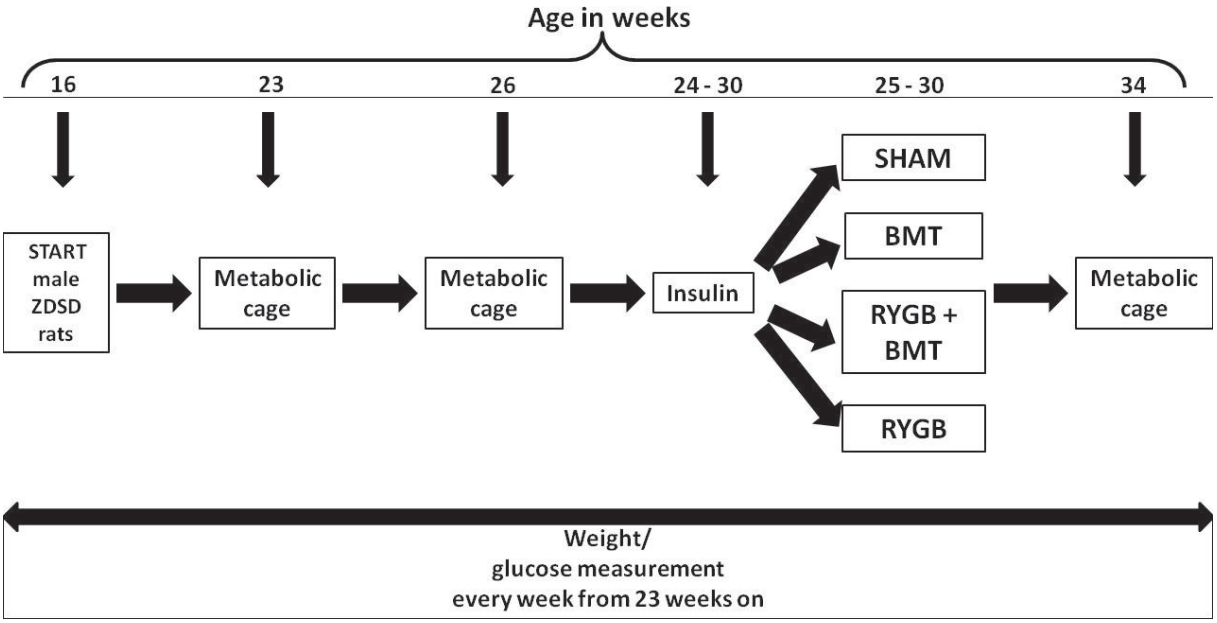
Aim 1 assessed the penetrance of the diabetic phenotype in ZDSD rats at the age of 26 weeks and examined, whether this tracked with level of injury biomarkers in the urine

Aim 2: quantified the relative impact of 3 intensive weight loss and metabolic control interventions on urinary kidney injury markers excretion in ZDSD rats.

2 Materials and Methods

2.1 Study design

The full study was approved by University College Dublin Animal Research Ethics Subcommittee (AREC) and work conducted under a governmental license from the Health Products Regulatory Agency-Ireland.



Schema 1General study plan on the ZDSD rats

The schema represents the timeline of the experiments and the subdivision of the ZDSD rats. Sham (positive control), BMT (best medical treatment), RYGB (Roux-en gastric bypass surgery).

2.1.1 Animal husbandry

All rats for the experiments were housed in the Biomedical Facility of the University College Dublin, Ireland. Forty four male Zucker Diabetic Sprague-Dawley rats (ZDSD) and seven Sprague-Dawley (SD) rats were obtained from CrownBio (Belgium), at the age of 14 weeks. The SD rats were used as the healthy control, because they are related to the ZDSD. All rats were housed (2/cage for the ZDSD and 3 – 4/ cage for the SD) in a controlled environment for humidity (35%), constant temperature (22 ± 1°C) and a 12 h:12 h light/dark cycle (lights on at 7 am). They were fed standard chow (Purina 5008 4.36 kcal/g, protein 23.6 %, fat 6.7 %) and water *ad libitum*.

2.1.2 Pre-surgery handling and division into experimental groups

From 16 weeks of age, all rats were weighed weekly. In addition from 23 weeks of age, plasma glucose was measured weekly.

At 25 weeks of age, 43 of the ZDSD rats were divided into 4 groups, equalized for their weight and plasma glucose and their KIM-1, Albumin, OPN and NGAL concentration in the urine. The numbers of rats in the two RYGB groups were higher, because of expected loss of animals during surgery.

a. Sham group (7 rats)
b. Medical treatment and 10 % weight loss (BMT) (8 rats)
c. RYGB group (14 rats)
d. RYGB group and medical treatment (RYGB(BMT)) (14 Rats)
e. Control (7 SD rats)

Post RYGB surgery, an adjustment of the animals per group was made, because some rats were lost after surgery. Two rats did not undergo surgery, but were added to the sham group. The final groups were:

a. Sham group (9 rats)
b. Medical treatment and 10 % weight loss (BMT) (7 rats)
c. RYGB group (9 rats)
d. RYGB group and medical treatment (RYGB(BMT)) (9 Rats)
e. Control (7 SD rats)

2.2 Interventions

2.2.1 Surgery

2.2.1.1 Sham surgery

At 25 weeks of age, the rats in the sham and BMT group received a sham surgery (laparotomy only). For seven days prior to surgery all animals were treated with long-acting insulin degludec (Tresiba®, Novo Nordisk A/S) according to their blood glucose. For dosage detail see appendix chapter 6.1. Rats were not fasted prior to surgery.
The animals were anesthetized using Isoflurane. They were laid out supine and a 2 cm midline abdominal incision was made. The stomach and intestine were made visible. The abdomen was then closed in two layers: First the abdominal muscles with a continuous and the skin with interrupted stitches. Vicryl 4-0 (Ethicon, Inc.) was used for both layers. The rat was put into a clean cage with paper and a warming pad to recover from anaesthesia. When fully recovered the rat were returned to a clean home cage.

2.2.1.2 RYGB surgery

The RYGB surgery was performed when the rats were 29-30 weeks old. Animals were treated for 7 days prior to surgery with long-acting insulin degludec (Tresiba®, Novo Nordisk A/S) according to their blood glucose. For dosage detail see appendix chapter 6.1. The day before surgery only a half dose was administrated. The animals were anesthetized using isoflurane. They were laid out supine and a midline abdominal incision was made. The proximal jejunum was divided 10 cm distal to the pylorus to create the biliopancratic limb and an alimentary limb. The biliopancratic limb was then sutured, via side to side jejuno-jejunoanastomosis, 30 cm proximal from the caecum to

the small intestine. The stomach was divided close to the gastro-oesophageal junction into a small pouch (max 3 ml volume) and a bigger pouch. The small pouch was stitched to the alimentary limb via end to side anastomosis. The remnant gastric pouch was closed using a continuous suture. Five ml of warmed, sterile saline (0.9%, 3.5 ml each side) were given intra peritoneal before closure. The abdomen was closed in two layers with continues stitches, using Vicryl 4-0 (Ethicon, Inc.) for the linea alba and another Vicryl 4-0(Ethicon, Inc.) for a modified intracutan stitch to close the skin.

2.2.1.3 Post-surgical care

Post surgery the animals were housed for 3 days without bedding, to prevent aspiration. They received five days of prophylactic treatment with enrofloxacin (7.5mg/kg) and one to three days buprenorphine (0.05mg/kg), as required. Only water was available *ad libitum* at the day of surgery. On day one and two post surgery the rats received 20ml respectively 30 ml of liquid diet (Ensure® Plus, Vanilla, 1.5kcal/ml, 16.7% protein, 29.5% fat, 53.8% carbohydrate) to facilitate passage through the oedematous surgery part of the gastrointestinal tract and reduce damage. On day three they were returned onto normal bedding and were fed wet mash. On day seven post surgery all rats returned to normal chow (Purina 5008). Five rats received normal chow already on day 6 post surgery, because they haven't been eating the wet mash well. During the first ten days post surgery the ZDSD were monitored daily for weight loss, food and water intake, pain and sings of infection. If the weight loss was > 30% the animal was euthanized. For more details regarding pain scoring see appendix 6.3.
Some rats showed hypersalivation, porphyria and reduced food intake >14 d post surgery. The normal chow was substituted for two days by a rat smoothie (Purina 5008 crushed, and soaked in water, 5 ml of Ensure® Plus and water to adjust the viscosity).

2.2.2 Food restriction and medical treatment of BMT group

The food of the BMT rat was reduced, when 29 weeks old. The goal was 10% weight loss in three weeks. Initially all received 16 g of standard chow (Purina 5008). The weight was controlled every 3 days and the amount of food given, was adjusted +2 g, 0 or –2 g, according to their weight change. In addition they received metformin hydrochloride (300mg/kg). After 10 days blood glucose was measured. All BMT rats received then in addition to metformin also rosuvastatin (10mg/kg), fenofibrate (100mg/kg) and ramipril (1mg/kg). When the 10% weight loss was achieved liraglutide treatment was started. The dose was titrated over 14 days to 1mg/kg subcutaneous. In this period water intake was monitored to avoid dehydration. At day 11 the Liraglutide treatment was interrupted due to massive weight loss. All BMT rats received then 30g of chow/day to regain weight towards target. Liraglutide was reinitiated at 0.2mg/kg.
metformin, rosuvastatin, fenofibrate and ramipril were given orally. The tablets were ground to a powder using a mortar and pestle, dissolved in water and then distributed over the rats daily chow ration.

2.2.3 Best medical treatment of RYGB(BMT) group

After RYGB surgery, the rats were given few days to recover. When all rats from the RYGB(BMT) group were at least 7 day post surgery, they received metformin

hydrochloride (300mg/kg) with 20g standard chow. Two days later rosuvastatin (10 mg/kg), fenofibrate (100mg/kg) and ramipril (1mg/kg) were added. Three weeks post surgery the chow ration was increased to 25 g/day, to ensure *ad libitum* food intake. Metformin, rosuvastatin, fenofibrate and ramipril were given orally. The tablets were grounded to a powder using a mortar and pestle, dissolved in water and then distributed over their daily chow ration.

2.3 Procedures

2.3.1 Blood glucose

Plasma glucose was measured using Contour® Glucose strips (Bayer Ltd, Dublin Ireland). Blood was obtained by puncturing the lateral tail vein and measured using the Bayer Contour Blood Glucose Meter. The maximum detection limit for glucose was 33.3 mmol/L. Values above that could not be measured and were thus included in statistical analyses as 33.3 mmol/L.

2.3.2 Blood plasma

Blood plasma was obtained by puncturing the lateral tail vein at the most distal point. The blood was collected by allowing it to drop into a 300 µl heparin coated tube. The blood was centrifuged for 5 min with 2000 rpm. The plasma was pipetted into an Eppendorf tube and then stored at -20° C till analyzed.

2.3.3 Metabolic cages

The ZDSD and SD rats were put into metabolic cages to collect urine when 23 and 26 weeks old. In addition urine was collected four weeks after initiating the intervention of each study group (when 33 – 34 weeks old). The rats spent 15 -18 h in the metabolic cage. The urine was used to measure albumin, NGAL, OPN, KIM-1 and creatinine, to assess kidney damage. In addition food intake, urine production and water intake were measured when 26 weeks old.

The collected urine was centrifuged for five min with 2000 rpm and then pipette into Eppendorf tubes for storages in the freezer at -20° C.

2.3.4 24 hour food intake

The food intake was measured at 7:00, 13:00, 19:00 and again at 7:00 of the following day. The measurement was performed when all rats were 34 weeks old.

2.4 Definition of diabetes

An afternoon plasma glucose of < 7 mmol/L was considered normal, 7.7 mmol/L – 11.0 mmol prediabetic and >11.0 diabetic. Plasma glucose of > 7 mmol/L was previously classified as prediabetic in ZDSD rats by Peterson, 2015 (115). Standard criteria in humans considers blood glucose > 11.0 mmol as diagnostic of diabetes and was used as a cut off in this study.

2.5 Kidney injury marker tests

2.5.1 Kidney injury biomarker ELISA

The Kidney Injury Panel 1 (rat) (Mesoscale, Rockville, USA) was used to measure KIM-1, NGAL, OPN and albumin in collected urine via electrochemiluminescence (ECL). The samples were diluted 1:5 with Diluent 29. The ELISA was done according the manufacturers instruction. After adding the read buffer, the plate was placed immediately into MESO QuickPlex SQ 120 and the measurement started. The data was visualized and analyzed using Discovery Workbench 4.0. The hourly excretion rate of each biomarker was calculated as follows:

$$\frac{Concentration\ Biomarker\ meassured\ with\ ELISA\ [ng/ml]}{time\ in\ metabolic\ cage\ [h]} \times total\ urine\ produced\ [ml]$$

Values under detection limit were included as 0ng/h into the statistic.

2.5.2 Urinary creatinine ELISA

The QuantiChromTM Creatinine Assay Kit (DICT-500) (BioAssay systems, US) was used to measure creatinine in urine and blood samples. The samples were not diluted. A clear bottom 96-well plate was used for the assay. The optical density was read immediately after adding the working reagent and again after five min at 490 – 530 nm (peak 510nm). For the quantification ClarioStar (BMG Labtech) was used. The detection limit was 0.1 mg/dL (8 µM).

2.5.3 Creatinine clearance

The creatinine clearance was calculated as following

$$\frac{urine\ volume\ [ml] \times creatinine\ concentration\ in\ urine\ [mg/dl]}{creatinine\ concentration\ in\ serum\ [mg/dl] \times time\ in\ metabolic\ cage\ [min]}$$

Two samples were excluded for the calculation, because the plasma creatinine values were more increased than expected and blood contamination was suspected.

2.6 Statistical analysis

GraphPad Prism 6 and Excel were used for all statistical analysis. Data are described with mean and standard deviation (SD) or median and 25th to 75th quartiles, if not mentioned otherwise. The box plots show the median the 25th to 75th quartiles and the minimum and maximum values.
Significance was shown in the graphs with * $p < 0.05$, ** $p < 0.01$ ***$p < 0.001$ and **** $p < 0.0001$

At the age of 26 weeks the 44 ZDSD rats were divided into quartiles, according to their blood glucose. The animals from the upper glucose quartile (UGQ) were compared to the ones from the lowest glucose quartile (LGQ). Results were reported with median, 25th and 75th quartiles. The two groups were tested with a Mann-Whitney U test for differences in albuminuria, NGAL, OPN and KIM-1 secretion into urine, creatinine

clearance, food and water consumption and urine production. A p-value < 0.05 was considered significant. Only 11 out of total 22 LGQ and UGQ animals were used to measure the creatinine clearance, because most of the plasma samples were haemolytic and would have interfered with the creatinine result. Two values from the LGQ creatinine clearance were eventually excluded from the graph and statistic, because blood contamination was expected.

To assess the correlation between urinary kidney injury biomarkers and blood glucose in ZDSD rats a Spearman's rank correlation was performed. A $p < 0.05$ was significant. In addition the albumin excretion rate (AER) was correlated with NGAL, OPN and KIM-1 excretion rate.

Weekly measurements of weight and blood glucose are shown with mean and standard error mean (SEM). Blood glucose was not measured when the animals were 27- 30 weeks old, because insulin treatment and surgery would have influenced the results. The mean weight and blood glucose, at 34 weeks of age, is calculate from only 6 rats in the BMT group and 8 rats in the RYGB(BMT) group. Differences in weight loss between intervention groups were assessed with an ANOVA, two tailed.

Weight loss post surgery was shown with mean and SD. Weight gain of the rats in the RYGB and RYGB(BMT) was compared using a t-test, two tailed. A p-value < 0.05 was considered significant.

The results from the 24 food intake are shown with median and 25th to 75th quartiles, since the data was not normally distributed. One ZDSD rat in the RYGB group was excluded from the graph and the statistics, because it did not eat during night. Hypersalivation with transient anorexia was expected. Including this rat, would have compromised the result. Because the data was not normally distributed, a Kruskal Wallis test, two sides, was performed to compare the median 24 h food intake of the sham, RYGB and RYGB(BMT) rats. A p-value of < 0.05 was considered significant.

Blood glucose, and the kidney injury biomarkers were compared pre and 4 weeks post intervention, to assess the success of them. A Wilcoxon test was used to compare the two outcomes. Results with $p < 0.05$ were considered significant.

3 Results

3.1 Subdivision of ZDSD rats into prediabetic and diabetic

Forty-four ZDSD rats were categorized at the age of 25 weeks according to their blood glucose in normal blood glucose (< 7mmol/L), prediabetic (7 mmol/L – 11.0 mmol/L) and diabetic (> 11mmol/L). The blood glucose mean of the control (SD rats) at 26 weeks was 5.3 mmol/L. Fifty-two percent of the ZDSD rats were considered prediabetic and 40.9% diabetic. Only 6.8% of ZDSD had normal blood glucose levels, see Figure 1.

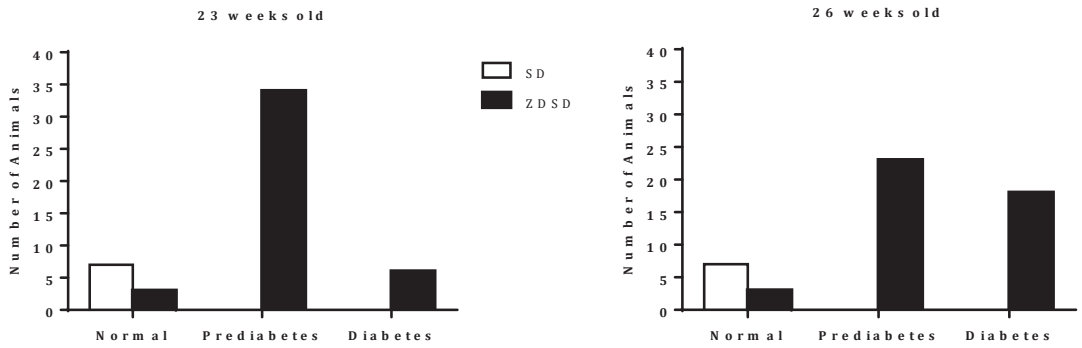


Figure 1 ZDSD rats are predominately prediabetic by 26 weeks
The graph shows the number of ZDSD rats being diabetic and prediabetic at 23 weeks and 26 weeks compared to the SD rats. The total number of animals of ZDSD rats was n = 43 at 23 weeks and n = 44 at 26 weeks.

3.2 Comparison between ZDSD rats having high and low blood glucose

In order to state if high glucose has an impact on the body weight, 26 weeks old ZDSD rats were divided into quartile according to their blood glucose. The lower glucose quartile (LGQ) group was compared to the upper glucose quartile (UGQ) group. The median (25^{th} – 75^{th} quartile) blood glucose of the LGQ was 7.8 mmol/L (6.8 - 8.1) and from the UGQ 19.7 mmol/L (16.2 - 23.3). According to the blood glucose levels all rats from the UGQ were diabetic. The rats in the LGQ were either non diabetic or prediabetic. The rats from the UGQ group were on average heavier than the ones from the LGQ group with 557g (545 - 573) versus 541g (515 - 553).

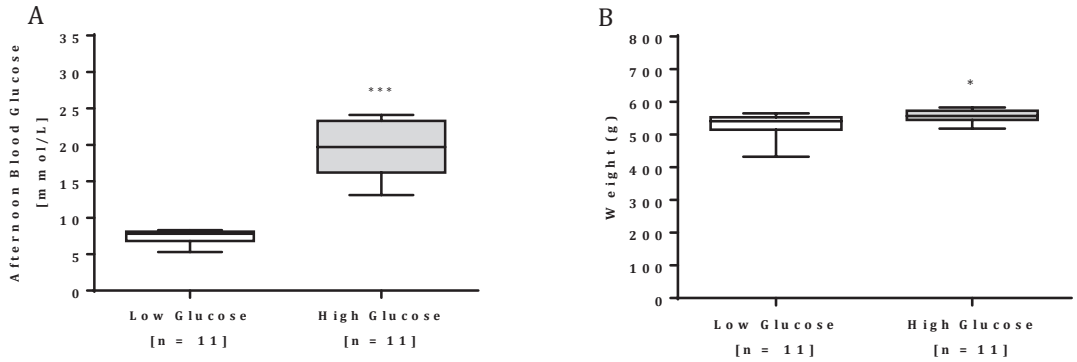


Figure 2 Blood glucose and weight comparison between the lowest and highest quartiles
A: Blood glucose levels were compared between ZDSD rats in the LGQ and the UGQ. B: Likewise the bodyweight was compared. Mann-Whitney U, two tailed, *** = p-value < 0.001 and * = p-value < 0.05

3.2.1 Characteristics of the diabetic phenotype in ZDSD rats, when 26 weeks old

The ZDSD from the UGQ and the LGQ were left in the metabolic cages over night for 17.5 hours. The water and food intake and the urine production were compared. The rats from the UGQ showed polyuria, polydipsia and polyphagia, symptoms of diabetes, in accordance of their blood glucose. The rats from the UGQ produced significantly more urine than the rats from the LGQ, 34 ml (20-64) versus 11 ml (8 – 13, ($p < 0.001$). They ate 24.1 g (22.6 – 31.2) and 16.1 g (14.2 – 19.2) respectively and drank 52.7 g (39 – 64.0) versus 24.5 g (21.4 – 28.3) (both $p < 0.0001$), see Figure 3.

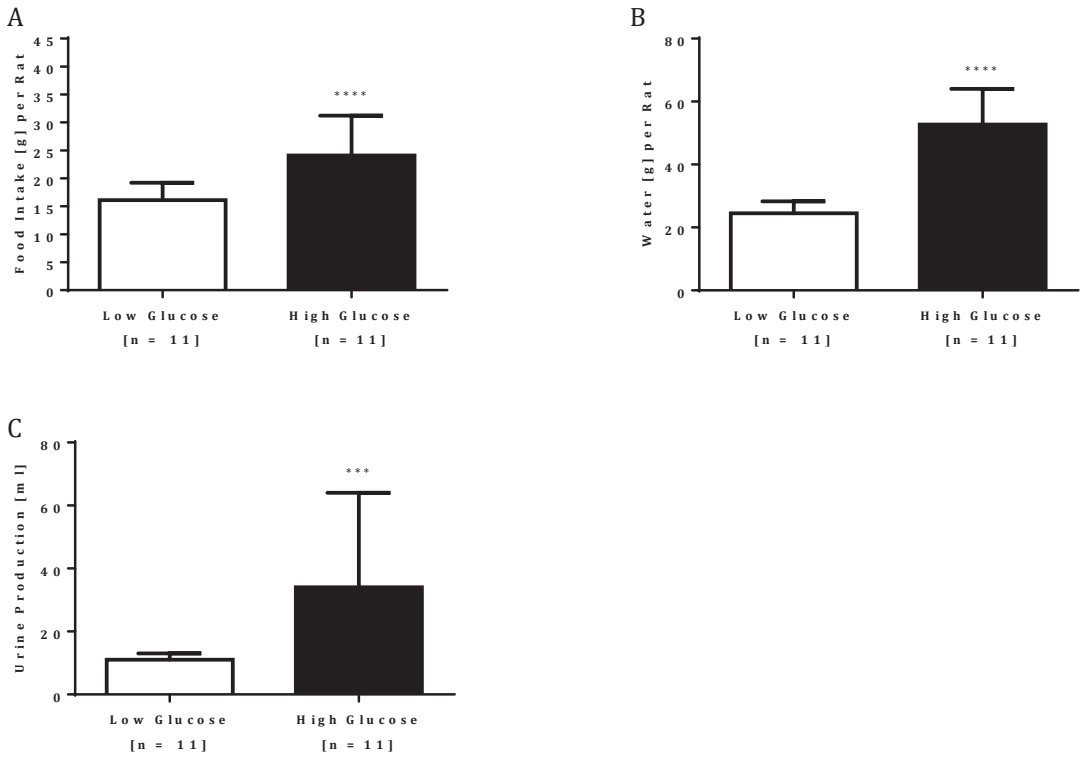


Figure 3 Urine production, food and water intake in the rats with high and low blood glucose.

A: The graph shows the medians of the food intake, B: water consumption and C: urine production of the ZDSD rats in the UGQ versus the LGQ. **** = p-value < 0.0001 and *** = p-value < 0.001, Mann-Whitney U, two tailed.

3.2.2 ZDSD rats with high blood glucose excrete higher levels of kidney injury markers into the urine

3.2.2.1 High blood glucose correlates positively with degree of albuminuria

Correlation between afternoon blood glucose measurements of the ZDSD rat's urinary albumin excretion rate at 23 and 26 weeks was investigated. At 23 weeks no correlation could be found between the two parameters, $p > 0.05$. At 26 weeks the plasma glucose level correlated positively with the urinary albumin excretion rate ($r = 0.47$, $p = 0.0014$). If the ZDSD rats from the UGQ and LGQ were compared, UGQ rats showed an increased albumin excretion rate with 156 µg/h (109 – 400) compared to 66.0 µg/h (58 -116) in the LGQ, p-value < 0.01). The median albumin excretion rate also differed between the control (SD rats) and the LGQ ZDSD rats, $p < 0.001$).

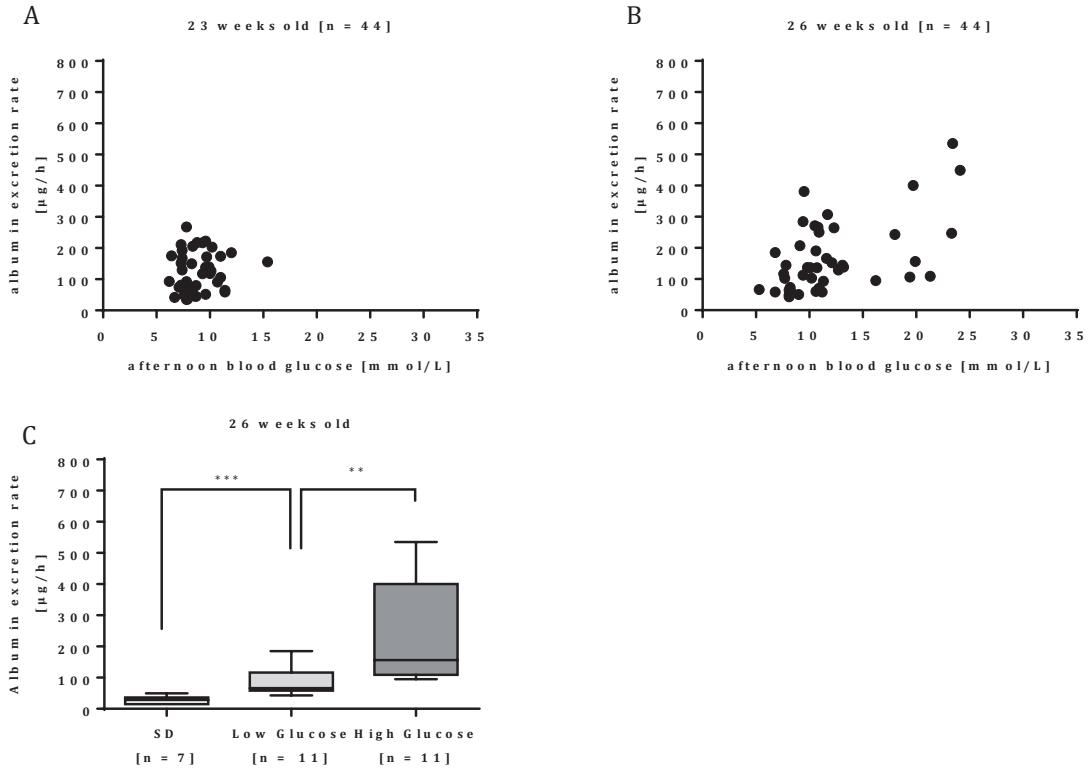


Figure 4 Correlation of blood glucose with urinary albumin excretion

Graph A and B show the correlation of the urinary albumin excretion rate with the afternoon blood glucose in the ZDSD rats at 23 weeks, Spearman's correlation Rank test, two tailed. Each dot represents an individual. Graph C shows the median of the albumin excretion rate in the control (SD rats) and the ZDSD rats from the UGQ and LGQ at 26 weeks. ** = p-value < 0.01, *** = p-value < 0.001, Mann-Whitney U test, two tailed, LGQ rats versus SD or versus UGQ rats.

3.2.2.2 Urinary NGAL excretion correlates with blood glucose in 26 week old ZDSD rats

The afternoon blood glucose measurements of the ZDSD rats were correlated with their urinary NGAL excretion rate at 23 and 26 weeks. At 23 weeks no correlation could be found between the two parameters, $p > 0.05$. At 26 weeks the plasma glucose level correlated positively with the urinary NGAL excretion rate ($r = 0.71$, $p < 0.0001$). ZDSD rats with a high blood glucose at 26 weeks had an increased urinary NGAL excretion rate compared to the ones with lower blood glucose with 189 ng/h (154.5 – 296.8) versus 92.9 ng/h (75.3 -118.4), $p < 0.001$. There was no difference in NGAL excretion rate between the control (SD rats) and the ZDSD rats with low blood glucose, $p > 0.05$.

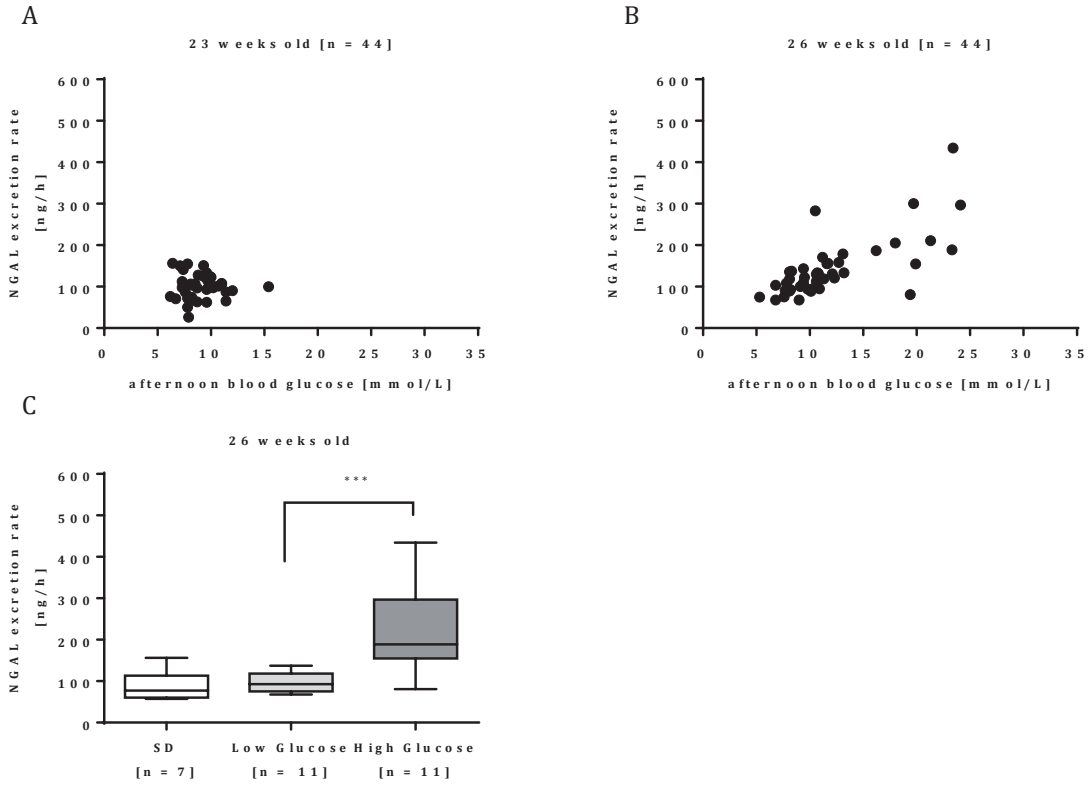


Figure 5 Correlation of blood glucose with urinary NGAL excretion

Graph A and B show the correlation of the urinary NGAL excretion rate with the afternoon blood glucose in the ZDSD rats at 23 weeks, Spearman's correlation Rank test, two tailed. Each dot represents an individual. Graph C shows the median of the NGAL excretion rate in the control (SD rats) and the ZDSD rats from the UGQ and LGQ at 26 weeks. *** = p-value < 0.001, Mann-Whitney U test, two tailed, LGQ rats versus SD or versus UGQ rats.

3.2.2.3 Urinary OPN excretion correlates with blood glucose in 26 week old ZDSD rats

The afternoon blood glucose measurements of the ZDSD rats were correlated with their urinary OPN excretion rate at 23 and 26 weeks. At 23 weeks no correlation could be found between the two parameters, $p > 0.05$. At 26 weeks the plasma glucose level correlated positively with the urinary OPN excretion rate ($r = 0.64$, $p < 0.0001$). ZDSD rats with a high blood glucose at 26 weeks had an increased urinary OPN excretion rate compared to the ones with lower blood glucose with 4.44 ng/h (3.06 -7.53) versus 1.83 ng/h, $p < 0.001$. Further, the urinary OPN excretion was significantly increased in the ZDSD from the LGQ compared to the controls, $p < 0.0001$.

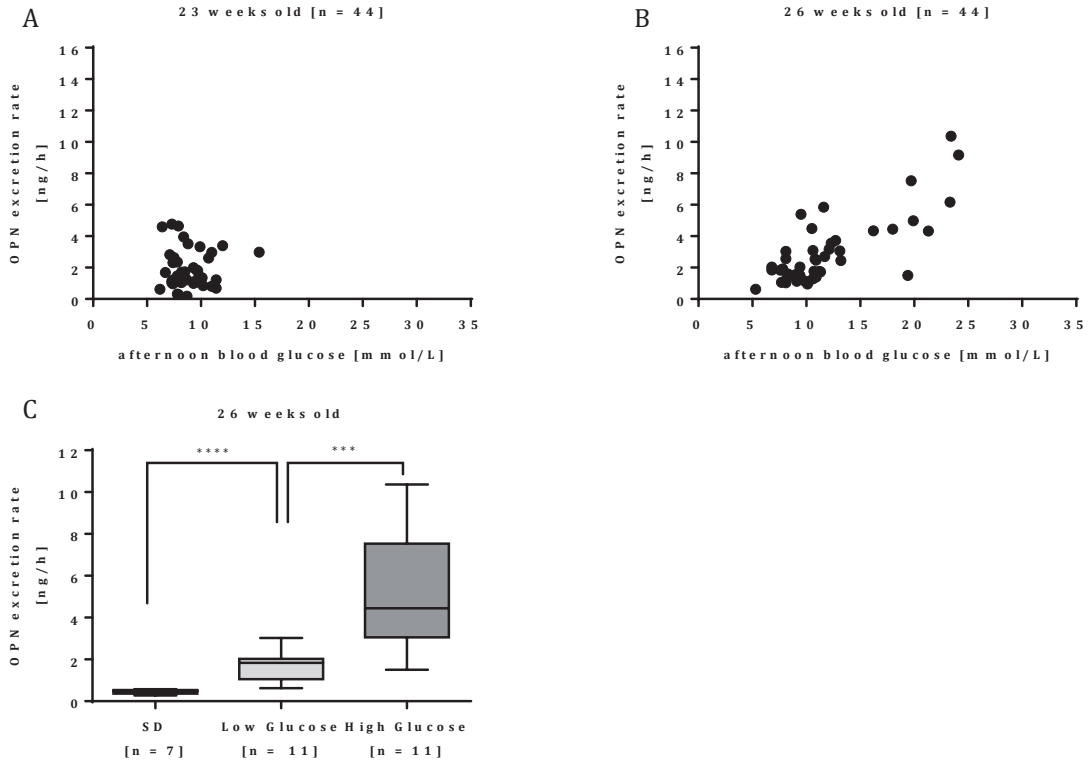


Figure 6 Correlation of plasma glucose with urinary OPN excretion

Graph A and B show the correlation of the urinary OPN excretion rate with the afternoon blood glucose in the ZDSD rats at 23 weeks, Spearman's correlation Rank test, two tailed. Each dot represents an individual. Graph C shows the median of the OPN excretion rate in the control (SD rats) and the ZDSD rats from the UGQ and LGQ at 26 weeks. *** = p-value < 0.001, **** = p-value < 0.0001, Mann-Whitney U test, two tailed, LGQ rats versus SD or versus UGQ rats.

3.2.2.4 Urinary KIM-1 excretion correlates with blood glucose in 26 week old ZDSD rats

The afternoon blood glucose measurements of the ZDSD rats were correlated with their urinary KIM-1 excretion rate at 23 and 26 weeks. At 23 weeks no correlation could be found between the two parameters, $p > 0.05$. At 26 weeks the plasma glucose level correlated positively with the urinary KIM-1 excretion rate ($r = 0.55$, $p < 0.001$). ZDSD rats with a high blood glucose at 26 weeks had an increased urinary KIM-1 excretion rate compared to the ones with lower blood glucose with 0.76 ng/h (0.72 – 0.94) respectively 0.55 ng/h (0.49 – 0.63), $p < 0.01$. There was no difference in KIM-1 excretion rate between the control (SD rats) and the ZDSD rats with low blood glucose, $p > 0.05$.

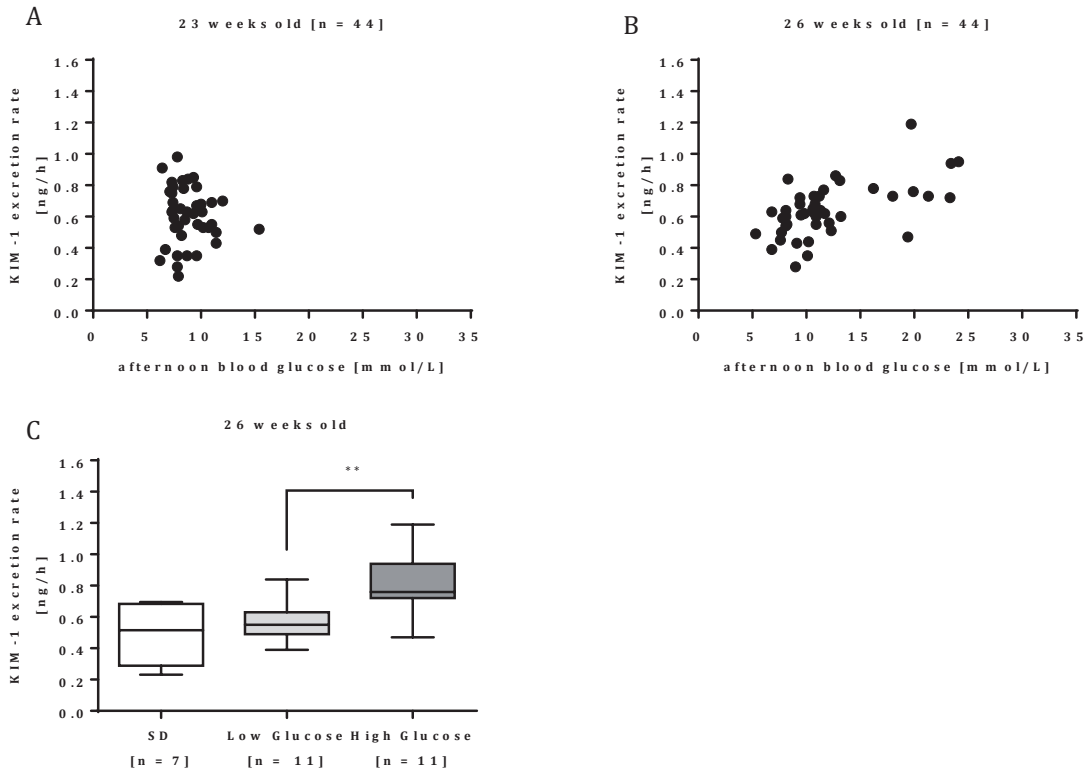


Figure 7 Correlation of plasma glucose with urinary KIM-1 concentration

Graph A and B show the correlation of the urinary KIM-1 excretion rate with the afternoon blood glucose in the ZDSD rats at 23 weeks, Spearman's correlation Rank test, two tailed. Each dot represents an individual. Graph C shows the median of the KIM-1 excretion rate in the control (SD rats) and the ZDSD rats from the UGQ and LGQ at 26 weeks. ** = p-value < 0.01, Mann-Whitney U test, two tailed, LGQ rats versus SD or versus UGQ rats.

3.2.2.5 Creatinine clearance does not differ in ZDSD rats with high and low blood glucose at 26 weeks

The creatinine clearance did not differ in depending on the afternoon blood glucose in the ZDSD rats at 26 weeks, p-value = 0.1.

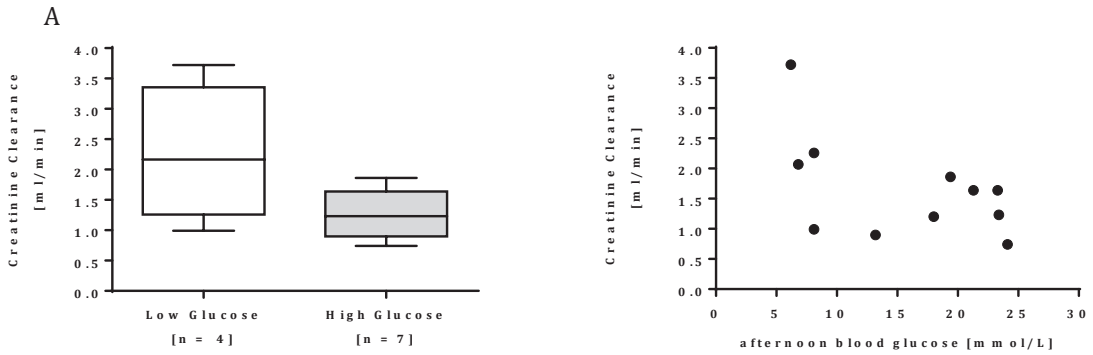


Figure 8 There was no difference in the creatinine clearance between the ZDSD rats in the upper and lower glucose quartile

In graph A the median of the creatinine clearance of the UGQ and LGQ at 26 weeks was compared with a Mann-Whitney U test, two tailed. Graph B shows the creatinine clearance in relation to the afternoon blood glucose of each individual of the UGQ and LGQ group.

3.2.3 Urinary NGAL, OPN and KIM-1 excretion correlate with urinary albumin excretion rate

A positive correlation was found between urinary albumin excretion rate and all the other tested kidney injury biomarkers in ZDSD rats at 26 weeks, p- value's all < 0.05. The strongest correlation was found between albumin and OPN (r = 0.54, p < 0.001) and the weakest with KIM-1 (r = 0.37, p = 0.0139), Spearman's rank correlation test.

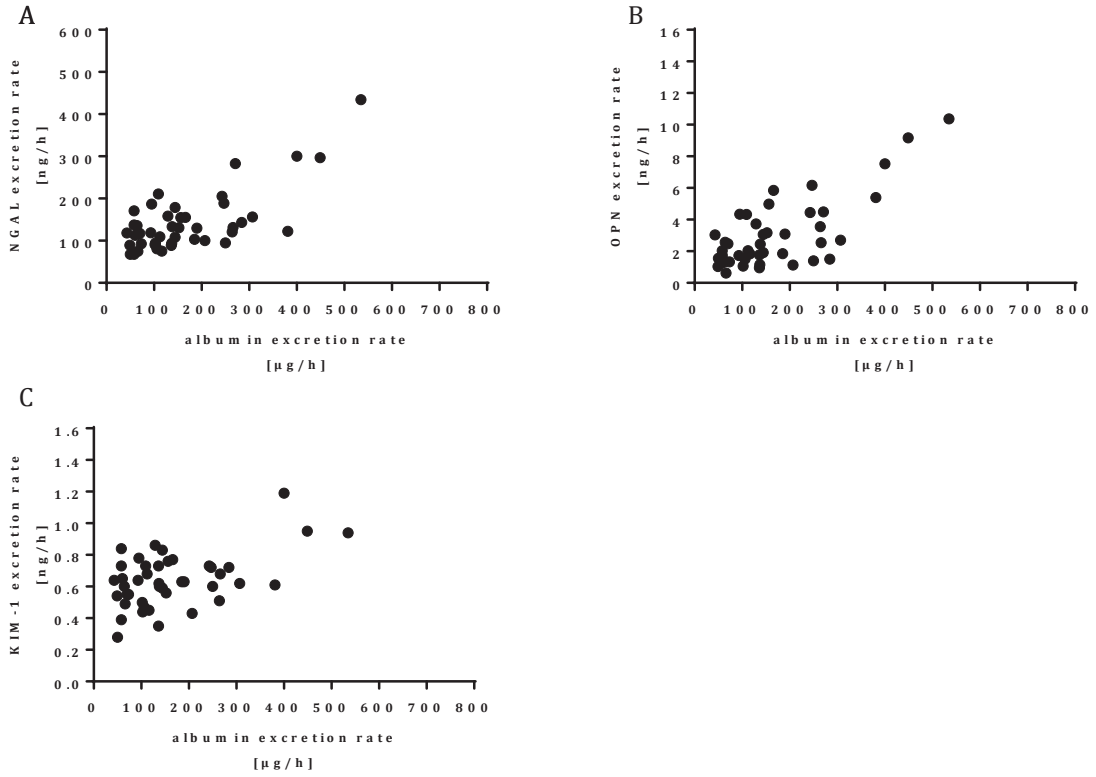


Figure 9 Correlation of urinary albumin excretion rate and NGAL, OPN and KIM-1
The graphs show the correlation between urinary albumin excretion and NGAL (A), OPN (B) and KIM-1 (C) in the 26 weeks old ZDSD rats, n = 44. Each dot represents an individual.

3.3 Haemorrhage is the main cause of death post RYGB surgery in ZDSD rats

None of the rats having sham surgery died.
The overall mortality from the RYGB surgery up to 10 days post surgery was 23%. Three animals died because of reactive haemorrhages < 24 h post surgery. One died within the first hour post surgery, the other two > 8 h - <24 h.

Three more rats had to be humanly killed 2 days, 5 days and 6 days post surgery, because of chronic bleeding or peritonitis, due to leak of the jejunojejunal or the gastrojejunal anastomosis. The cause of death of the third rat was not further investigated
A seventh rat was found dead 14 days post surgery. The cause of death was not further investigated. The animal had recovered well from surgery and had shown no adverse symptoms before death.

Three weeks post surgery another ZDSD rat was euthanized because of excessive weight lost. A ZDSD rat from the BMT group died when 33 weeks old, from an oesophageal mass. The case was not further investigated

Table 2 Cause of death post RYGB surgery

Time post surgery	Cause of death	No. of animals
< 1 h	Intra operative bleeding	1
< 24 h	Reactive haemorrhage	2
48 h	Chronic haemorrhage	1
5 d	Peritonitis	1
> 5 d	Unknown	2

3.4 Effect of medical treatment, weight loss and RYGB surgery on weight, blood glucose and kidney injury

3.4.1 Most weight loss occurs in the RYGB(BMT) group

All rats were weighed weekly. During the full study period, the SD rats gained more weight every week. The sham animals gained weight up to week 25. After sham surgery they lost up to 4.5% in one week, then no more surgery related weight loss was observed. When the sham animals grew older they, started to lose weight as their blood glucose increased, see Figure 10.

The food restriction for the BMT animals was started at 29 weeks of age. Over two weeks they lost 5.5 – 10.2 % of their initial body weight. Liraglutide treatment was titrated towards 1mg/kg but discontinued temporarily at a dose of 0.5mg/kg due to anorexia. As a consequence they had lost up to 19.2 % of their initial body weight, when 31 weeks old. To compensate the weight loss, the BMT rats were fed from then on 30g/d of standard chow to achieve the goal of 10% weight loss. With the weight gain, the blood glucose increased, see Figure 10. All ZDSD rats that had RYGB surgery lost initially weight. While the RYGB rats started to gain weight again 3 weeks post surgery, the rats also receiving medicaments continued losing weight, see Figure 10.

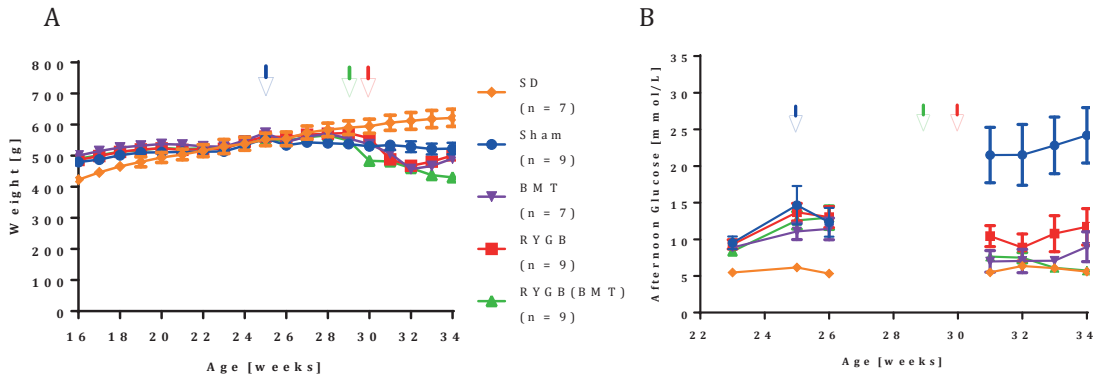


Figure 10 Development of the Body Weight and Blood Glucose from ZDSD and SD rats
The arrows indicate the week of surgery. Blue: sham surgery for the Sham and BMT group. Green: RYGB surgery for RYGB(BMT) and Red: RYGB surgery for RYGB group.
A: Body weight was measured weekly. Data are shown with mean and SEM bars.
B: Blood glucose was measured in the afternoon. When the rats were 27 till 30 weeks old, no blood glucose was measured. Data are shown with mean and SEM bars.

Most of the weight loss occurred in the first week post RYGB surgery as shown in Figure 11. The RYGB and RYGB(BMT) animals lost up to 20 % of their initial body weight in the first two weeks post surgery. However, three weeks post surgery the RYGB started to correct their body weight and gained some grams. The final weight loss of the BMT, RYGB and RYGB(BMT) was 89g ± 26.9, 78g ± 11.9 and 131g ± 17.2. Absolute numbers

correspond to -15.9% ± 1.8, -13.5% ± 6.3 and -23.0% ± 4.1 change in body weight post intervention for the BMT, RYGB and RYGB(BMT). The weight loss of all rats differed significantly between intervention groups, $p < 0.0001$, 1-way ANOVA, two tailed; only the weight of the RYGB and BMT group was the same. The main weight difference between RYGB and RYGB(BMT) formed 3-4 weeks post surgery, where it became significant, $p < 0.01$ t-test, two tailed. While the RYGB's gained weight, the RYGB(BMT) continued losing weight. The RYGB(BMT) ate less than the RYGB, $p = 0.0079$, Mann-Whitney U, two tailed, paired with the continuous weight loss, see Figure 11. The sham rat ate significantly more than the RYGB and the RYGB(BMT) with a median of 40g (27 – 60) compared to 29g (26 – 30) and 23g (17 – 25), $p = 0025$, Kruskal-Wallis. However the sham lost up to 4.5% ± 2.5 of their body weight within four weeks, see Figure 11.

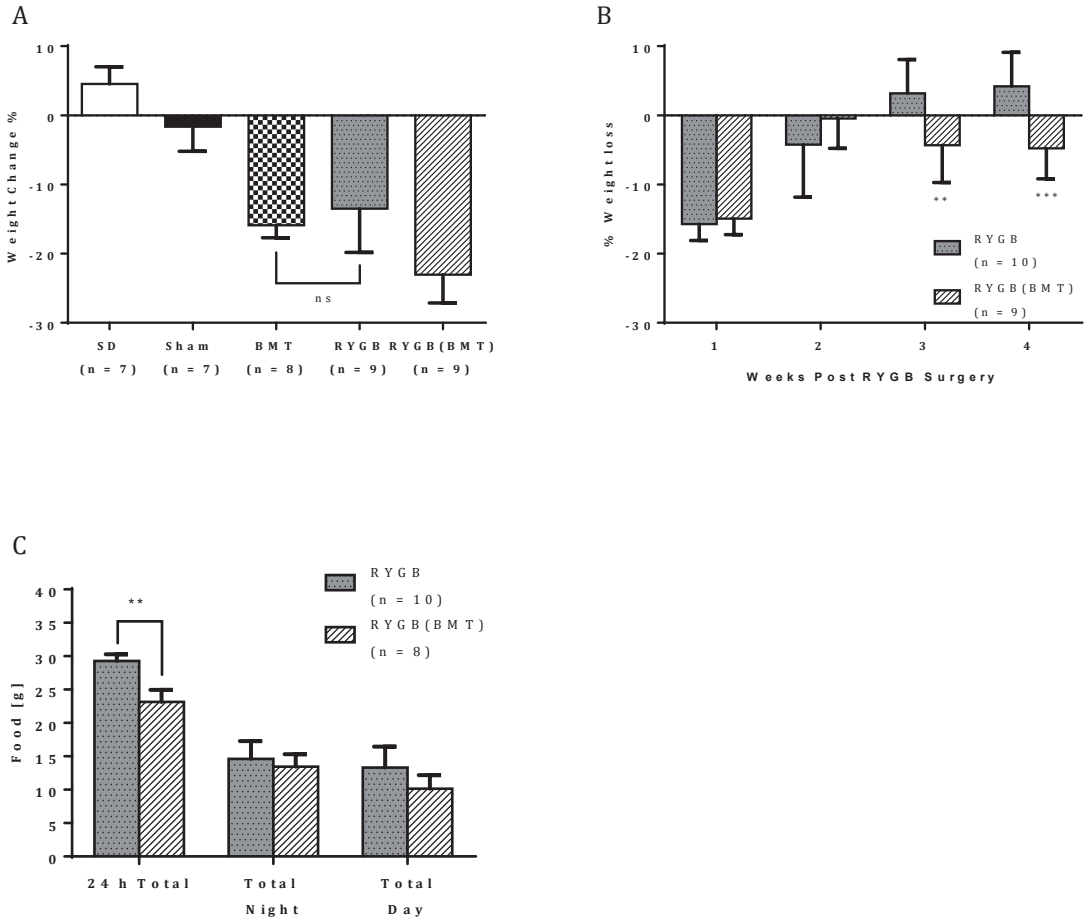


Figure 11 Body weight change 4 weeks post surgery and food intake
Graph A shows the relative weight changes of the ZDSD 4 weeks after RYGB surgery or weight loss paired with medical treatment compared to the SD and sham animals. B) Percentage of weight loss one to four weeks post RYGB surgery. C) Food intake in 24 hours. ns = non significant ** = $p < 0.01$, *** $p < 0.001$

3.4.2 RYGB(BMT) leads to the highest percentage of diabetes remission

Before the intervention all rats were at least prediabetic. Three ZDSD's in the sham group progressed from prediabetes to diabetes. Medical treatment and 15% weight loss (BMT) lead to an improvement of the hyperglycemia 4 weeks after treatment start. Diabetes remission to prediabetes was observed in all 3 previously diabetic rats. Fifty-seven percent of the ZDSD rats were non diabetic four weeks post treatment. Blood glucose improved in 2 out of 4 diabetic ZDSD rats post –RYGB surgery. Twenty-two percent of the ZDSD rats had normal blood glucose four weeks post RYGB surgery. The combination of RYGB surgery and medical treatment showed a complete remission of diabetes in 89% of the rats. Only one animal was still prediabetic 4 week post intervention. Over all the blood glucose sank significantly in the BMT and RYGB(RYGB) group, but was not significantly changed in the RYGB group 4 weeks post surgery, $p < 0.05$, Wilcoxon test, two tailed. In summary RYGB(BMT) showed the best results regarding hyperglycemia, followed by BMT and then RYGB, see Figure 12.

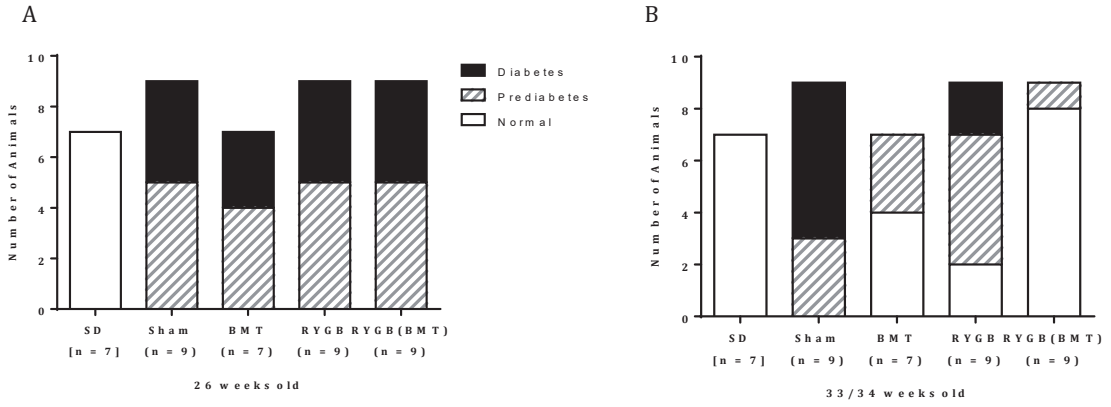


Figure 12 Number of rats having diabetes pre and post intervention

Graph A shows the number of animals being prediabetic or diabetic before the intervention, while Graph B shows the distribution of the rats four weeks post intervention.

3.4.3 Urinary kidney injury markers post intervention

3.4.3.1 BMT and a combination of RYGB and medical treatment improve albuminuria

Albumin was already significantly increased in the urine of the ZDSD rats compared to SD's, when 23 weeks old. This did not change when 26 weeks old as shown in Supplementary 3. Albuminuria worsened in the not treated sham group. When 33/34 weeks old, four sham animals lost more than 1250 µg/h albumin into the urine, one animal even more than 12500µg/h. Albuminuria improved significantly in the ZDSD rats receiving medical treatment and food restriction (BMT) with a median AER of 103µg/h (73 – 144) before treatment and 50 µg/h (31 – 64) after treatment, $p < 0.05$, Wilcoxon test, two tailed. RYGB surgery alone did not improve significantly the AER. However, some individuals in the RYGB group show improvement. All ZDSD receiving a combination of RYGB surgery and medical treatment improved the AER 4 weeks post intervention with 156 µg/h (93 – 246) pre treatment versus 28 µg/h (20 – 58) post treatment, $p < 0.05$, Wilcoxon test, two tailed.

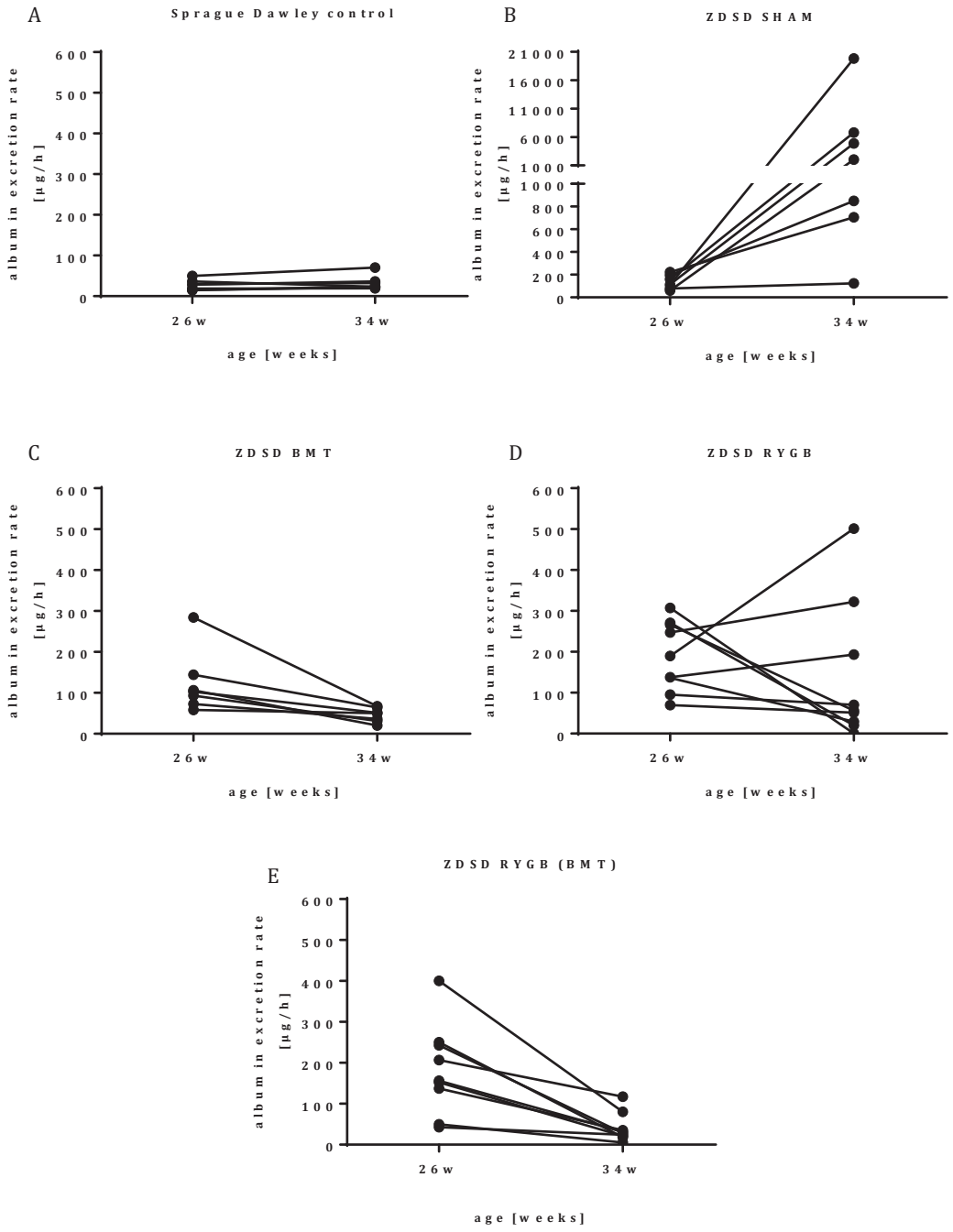


Figure 13 Urinary albumin excretion rate pre intervention (26 weeks old) and four weeks post intervention (33/34 weeks old)

Two combined dots represent the urinary albumin excretion rate of an individual pre intervention and 4 weeks post intervention. Graph A shows the healthy control (SD rats), graph B the untreated ZDSD rats (SHAM) and graph C-E the different intervention grorups.

3.4.3.2 Urinary NGAL excretion reduced after RYGB surgery

The NGAL concentration did not differ between the SD and ZDSD rats when 23 or 26 weeks old as shown in Supplementary 3. A significant increase of the median NGAL excretion into the urine could be observed 4 weeks post intervention (33/34 weeks) compared to the baseline (26 weeks) in the sham 345ng/h (141 – 783) versus 122ng/h (94 – 140) , $p < 0.05$, the BMT 314ng/h (248 – 366) versus 50ng/h (31 – 64), $p < 0.05$ and the RYGB(BMT) group 442ng/h (300 – 804) versus 184 ng/h (94 – 180), $p < 0.01$, Wilcoxon test, two tailed. The NGAL excretion did not differ in the healthy control (SD rats) and the ZDSD rats receiving only RYGB surgery four weeks post-intervention.

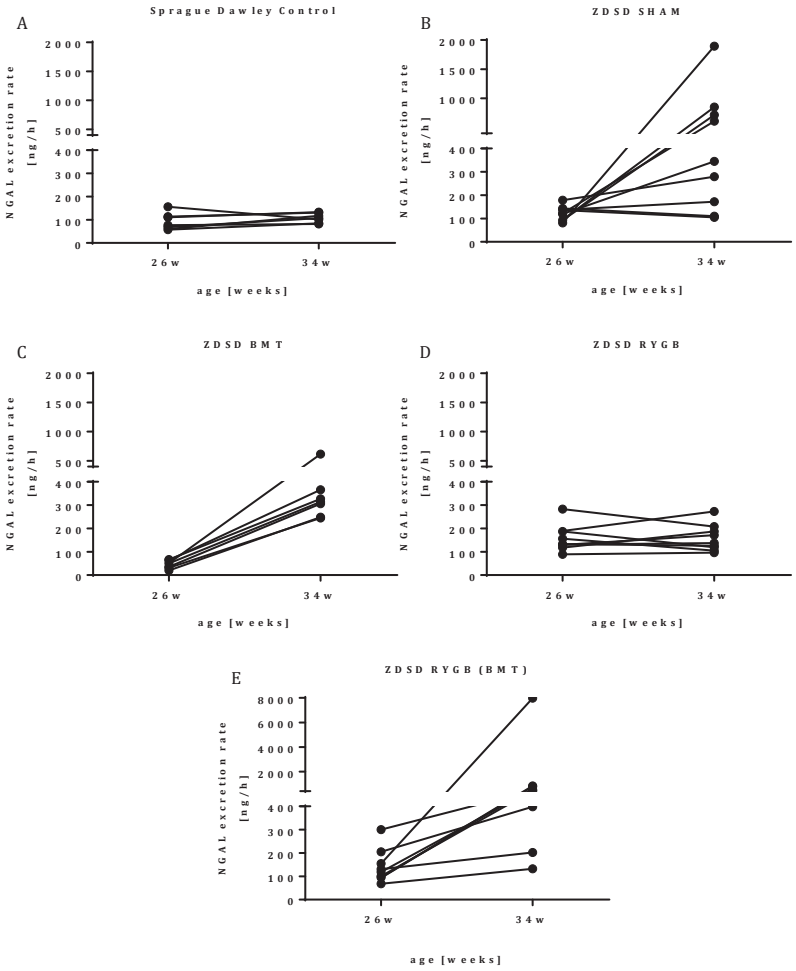


Figure 14 Urinary NGAL excretion rate pre intervention (26 weeks old) and four weeks post intervention (33/34 weeks old)

Two combined dots represent the urinary NGAL excretion rate of an individual pre intervention and 4 weeks post intervention. Graph A shows the healthy control (SD rats), graph B the untreated ZDSD rats (SHAM) and graph C-E the different intervention groups.

3.4.3.3 Urinary OPN excretion is reduced in the combination therapy of RYBG and medical treatment

Urinary OPN excretion rate increased in the healthy controls from 26 weeks to 33 weeks from 0.48 ng/h (0.37 – 0.53) to 0.82 ng/h (0.51 – 1.21), ($p < 0.05$). Urinary OPN did not differ significantly in the sham and RYGB(RYGB) group pre and post intervention. The SD rats and the ZDSD rats in the BMT and RYGB group had higher urinary OPN excretion 4 weeks post intervention, $p < 0.05$ Wilcoxon test, two tailed

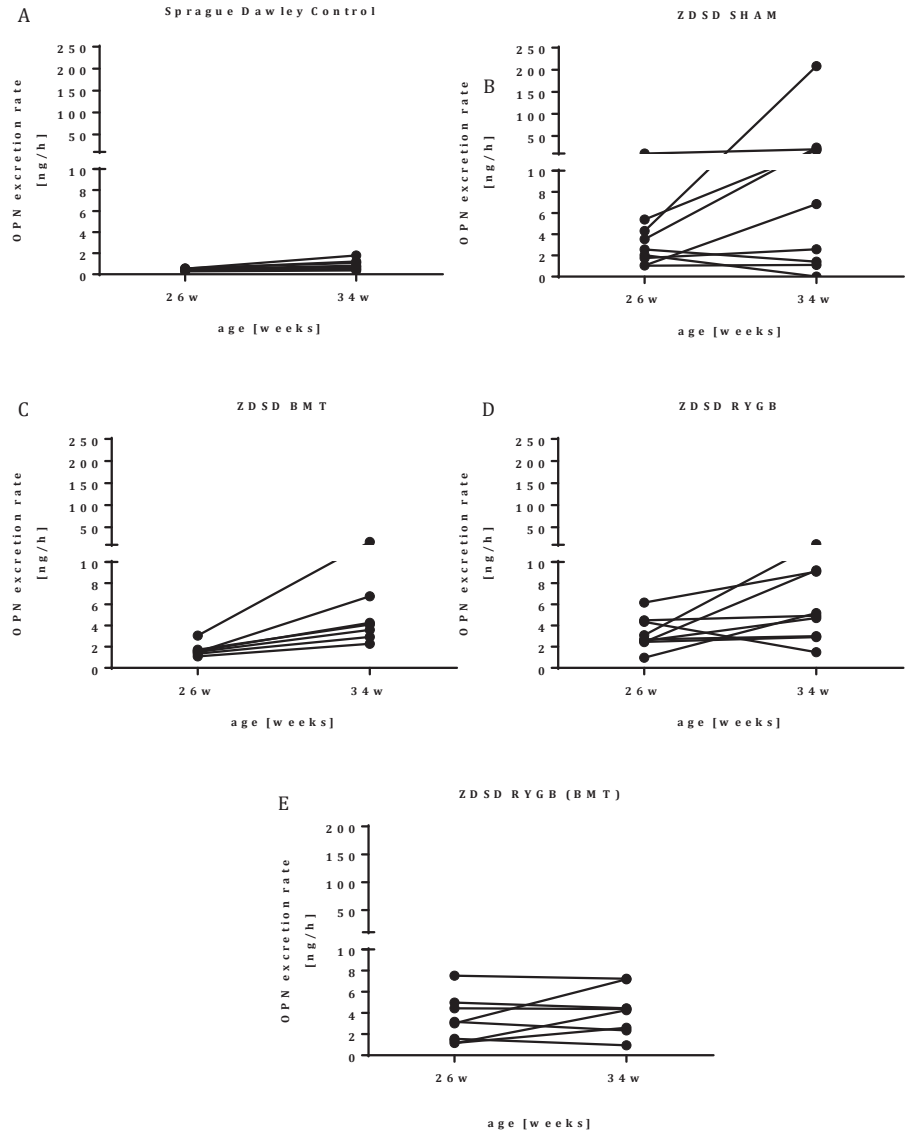


Figure 15 Urinary OPN excretion rate pre intervention (26 weeks old) and four weeks post intervention (33/34 weeks old)

Two combined dots represent the urinary OPN excretion rate of an individual pre intervention and 4 weeks post intervention. Graph A shows the healthy control (SD rats), graph B the untreated ZDSD rats (SHAM) and graph C-E the different intervention groups.

3.4.3.4 Urinary KIM-1 excretion is reduced when treated with a RYGB surgery, medical treatment or a combination of both

Urinary KIM-1 excretion rate was stable in the healthy control group over the full study period. No significance change in the urinary KIM-1 excretion rate could be found pre and post intervention for the SD, sham, BMT, RYGB or the RYGB(BMT) group, $p > 0.05$, Wilxoxon test, two tailed. There was no difference of the median OPN excretion rate between all the groups 4 weeks post intervention, $p > 0.05$, Kruskal Wallis test, two tailed. However there was a trend of increased KIM-1 excretion in the sham group and some rats from the RYGB group in the 4 weeks follow up period.

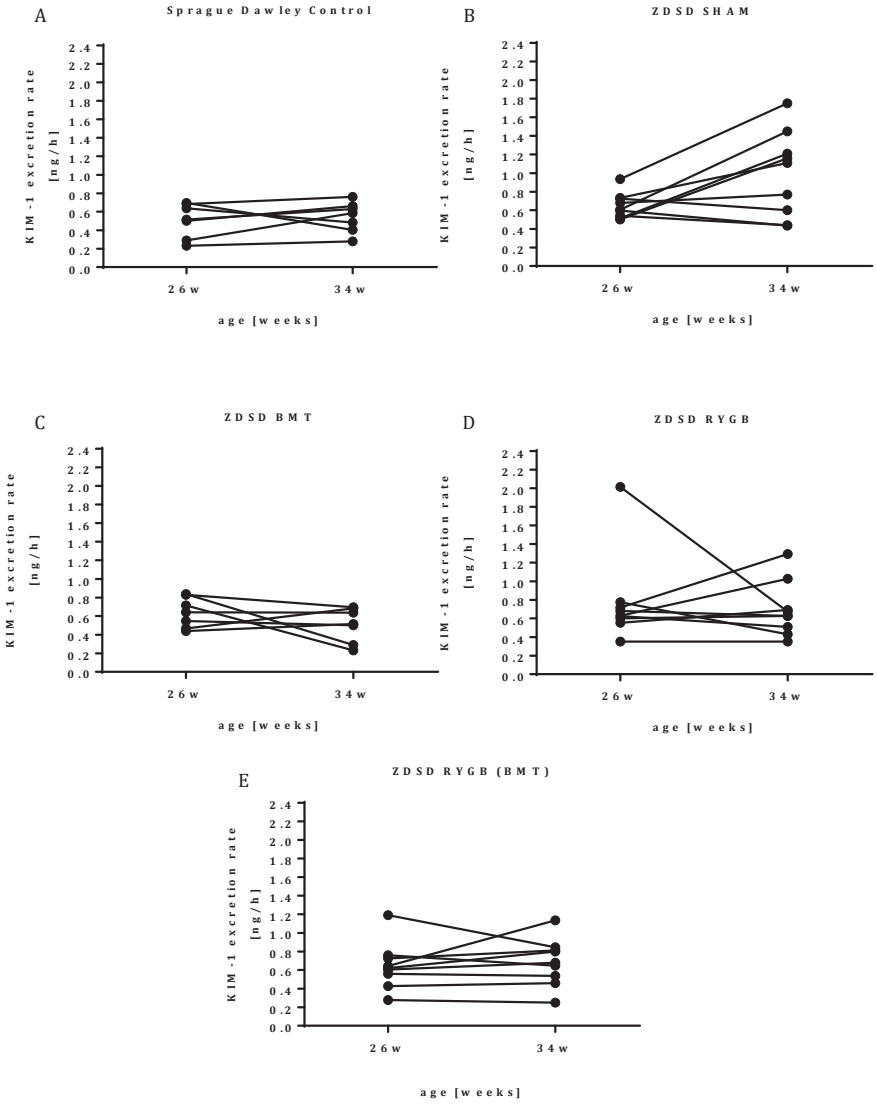


Figure 16 Urinary KIM-1 excretion rate pre intervention (26 weeks old) and four weeks post intervention (33/34 weeks old

Two combined dots represent the urinary KIM-1 excretion rate of an individual pre intervention and 4 weeks post intervention. Graph A shows the healthy control (SD rats), graph B the untreated ZDSD rats (SHAM) and graph C-E the different intervention groups.

3.4.3.5 Improvement of albumin excretion rate in medically managed diabetes leads to a increase of the NGAL excretion rate.

All ZDSD rats in the BMT and RYGB (BMT) group showed an improvement of the AER four weeks after intervention start. However they all showed an increase in the urinary NGAL excretion at the same time, see Figure 17.

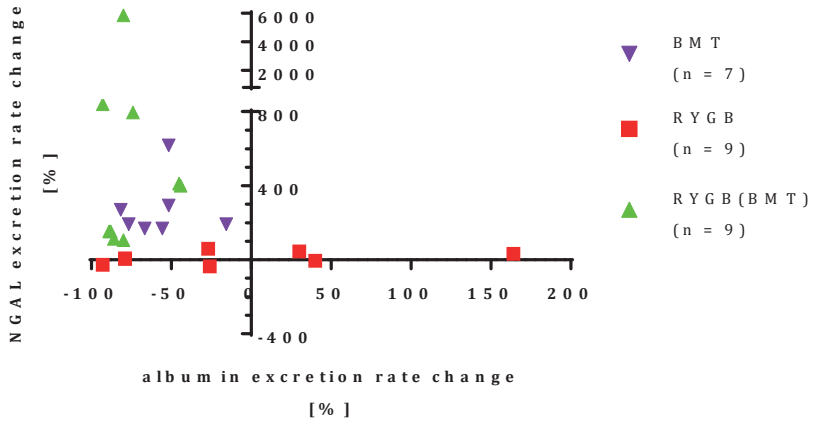


Figure 17 Albumin and NGAL excretion rate change 4 weeks post intervention
Each dot represents an individual.

4 Discussion

In this study different therapy forms to prevent diabetic kidney disease in T2DM were compared. ZDSD rats were used. They are a new rodent model for T2DM without a defect in the Leptin pathway. The healthy controls were the Sprague Dawleys (SD). During the study period significant variability in individual susceptibility regarding hyperglycemia, poyluria polydipsia, and kidney injury markers were observed within the ZDSD strain. These findings relative to findings in SD rats will be discussed in the first part of this chapter. The outcome of the interventions to prevent or delay diabetic kidney damage will subsequently be discussed.

4.1 Characterization of the ZDSD rat

In this study, a rat was considered diabetic, when their afternoon blood glucose was >11 mmol/L. This value was two times higher than the blood glucose measured in the SD rats. A rat was hyperglycaemic when blood glucose > 7mmol/L, which was 30% higher than the blood glucose levels in the control. Not all ZDSD rats were diabetic, when they were divided into the different intervention groups (sham, BMT, RYGB, RYGB(BMT). Most of them were prediabetic (52%) as demonstrated in Figure 1. The number of diabetic rats was lower than expected. According to Peterson et. al. (2015) the average fed blood glucose should be > 17 mmol/L by 25 weeks, whilst in our study this was 12.5 mmol/L. Interestingly, a weight gain stop was observed in the ZDSD rats of this study at the age of 20 – 22 weeks. This could have slowed down the development of hyperglycaemia and explain the difference in blood glucose between the two studies. The feed can be excluded as the cause. All rats from this and Petersons et al. (2015) were fed with Purina 5008, during the full study period. The weights were comparable and in both studies the rats were housed in pairs. The only difference was the time point when the blood was collected. Peterson et al. (2015) collected the blood early in the morning, while we collected it in the afternoon. However, at 24 week we collected blood in the morning and the obtained average blood glucose was significantly lower than the values at the age of 23 weeks. We observed a large variation in the blood glucose values. As a consequence, the ZDSD rats were divided into quartiles according to their blood glucose at 26 weeks. The animals in the upper glucose quartile were directly compared to those in the lower glucose quartile. Differences in weight, diabetic symptoms and kidney injury were investigated. The animals in the UGQ were all diabetic with a blood glucose of > 11 mmol/L. No animals in the LGQ were diabetic, but some were prediabetic. The ZDSD from the UGQ were heavier than those from the LGQ. This could be a possible explanation for the difference in blood glucose, Obesity is an important risk factor for diabetes (11). The weight difference possible evolved, because the rats in the UGQ ate more. Polyphagia is one on the main symptoms of diabetes mellitus. When diabetes mellitus progresses, not all the glucose is able to enter the cell, where it is metabolized to energy. The reason in T2DM is the increasing insulin resistance. As a consequence more food is needed to cover the energy waist.

The rats in the UGQ showed polyuria and polydipsia, typical diabetes symptoms, which were not present in the LGQ individuals. Polyuria is caused through the loss of glucose

into the urine. The osmolarity of the primary urine increases and less fluid is reabsorbed (osmotic diuresis). As a consequence the urine becomes less concentrated and more fluid is lost. To compensate for the dehydration water intake elevates.
Albumin, NGAL, OPN and KIM-1 were elevated in the urine of the UGQ rats compared to the LGQ ones. This findings are in accordance with a previous study, where diabetic ZDSD rats had increased kidney injury markers in the urine (72). The finding of polyuria and polydipsa in combination with increased kidney injury markers were expected. A recent study showed that already small changes in the albumin excretion rate within one year can worsen the prognosis to develop chronic kidney disease before the presence of microalbuminuria (116). NGAL and KIM-1 are associated with tubulointerstitial kidney damage. The NGAL excretion rate increases with the severity of the renal injury (117). As expected, our findings show that kidney damage is more advanced in the ZDSD rats with high blood glucose. We can agree with Peterson et al. (2017) that ZDSD rats are a suitable model to study DKD.

Interestingly at 23 weeks albumin and OPN were elevated in the urine of ZDSD rats compared to the control rats (SD rats). This result differs from what was previously published. Peterson et al. (2017) found no difference in the albumin excretion between the SD and ZDSD rats before 26 weeks. Instead they measured an increased KIM-1 excretion compared to the control already at 22 weeks. Recent studies have shown that human patients with only low levels of albumin excretions (< 30mg/24h) have an increased risk of hypertension and mortality (118). In fact, albuminuria seems to worsen in association with hypertension and metabolic changes (119). In healthy US veterans it has been demonstrated that the change in the amount of albumin excreted within one year, can be associated with the outcome of CKD. None of the tested individuals showed micro or macroalbuminuria at the beginning of their study (116).

In our study we found a significant difference in the urinary OPN excretion between the SD and ZDSD rats, probably because of ongoing kidney damage in the ZDSD rats. Al-Malki et al. (2014) found an association between albuminuria, podocyte damage and urinary OPN excretion(120). This suggests that the ZDSD rats suffer from podocyte loss and decreased glomerular function. However, Yamagucci et al. (2004) found that urinary OPN did not correlate well with the degree of kidney damage. He suggested that plasma OPN would be a better analyte.(121).

Because of blood contamination and haemolysis only few samples could be analyzed for the creatinine clearance. No difference was found between the animals from UGQ and LGQ. Expected was a higher or normal creatinine clearance. It would be an approximation for the GFR. In early kidney damage the GFR is increased without albuminuria. Later the GFR goes back to a normal level, while microalbuminuria is present, before the GFR decreases more with the severity of the kidney damage. But recent studies have demonstrated that the GFR can be increased or already decreased before microalbuminuria is measured (5, 6).

4.2 Correlation of urinary kidney injury markers and glycaemia

The urinary excretion rate of kidney injury markers (albumin, NGAL, OPN, KIM-1) was compared to the degree of hyperglycaemia. The goal was to asses if hyperglycaemia correlates with kidney injury. Our results showed that albumin, NGAL and OPN and KIM-1 correlated with the degree of hyperglycaemia, when the animals were 26 weeks old. These findings suggest that massive hyperglycaemia causes more kidney damage than normal or only mild hyperglycaemia. Interestingly there was no correlation between any of the kidney injury markers and hyperglycemia, when the animals were 23 weeks of age. Suggesting that hyperglycemia has to be present for some time before kidney damage ensues. Huang et al. (2015) found an association between the albumin creatinine ratio (ACR) and fasting blood glucose. However, when the findings were adjusted to HbA1c the correlation was not anymore present (122). Increased HbA1c is the consequence of long term elevated blood glucose; supporting our theory that hyperglycaemia has to be present for a period before kidney damage occurs. It highlights the importance of treating at hyperglycaemia as early and adequately as possible to prevent progression to ESRD (31).

The strongest correlation was found between urinary NGAL and glycaemia. Wang et al. (2006) published a correlation between glycaemia and serum NGAL (123). He even proposed NGAL as an independent risk factor for insulin resistance and hyperglycemia. However Elkhidir et al. (2017) could not find any correlation between serum NGAL and glycemic control (124). But in our study we showed, that there is a correlation with urinary NGAL and hyperglycaemia.

The elevation of all four kidney injury parameters suggest that not only one part of the kidney is affected by hyperglycaemia, but the full kidney. Albuminuria is traditionally associated with endothelial dysfunction and increased intraglomerular pressure. NGAL is secreted mainly in the thick ascending limb of the Henle loop, distal tubulus, and the collecting duct, while KIM-1 is secreted in the proximal tubules in case of kidney injury (125). OPN is expressed by the distal tubular epithelial cells. In the case of kidney damage, it can also be up-regulated in the glomeruli and by the macrophages found in the parenchyma (106, 107).

In this study the excretion rate of NGAL, OPN and KIM-1 correlated with the AER. So far, albuminuria has been the gold standard to assess kidney damage. NGAL has been proposed as a marker for acute kidney injury, but can also be elevated in chronic kidney disease (81). With our results we can contribute that NGAL is a good marker for DKD. KIM-1 can be elevated in DKD and was previously proposed to be a possible marker for kidney injury (111). Supported by the findings of this thesis OPN can be a possible marker for DKD and kidney injury. However, to assess the real value of each of these markers in connection with the effective degree of kidney injury further studies are needed.

4.3 Mortality rate and complications

The main cause of death during or < 48 h post surgery was bleeding. The mortality rate was 23% this was higher than expected. Most studies with RYGB surgeries report a maximum mortality of 20% (126). Only Inoue et al. (2007) published a higher mortality of 35% (127). A common reason for increased mortality is the surgeon. However in our case the surgery was performed by an experienced person. More likely an intrinsic susceptibility to bleeds in the ZDSD rat is the problem themselves. ZDSD rats become hypertensive by 9 weeks (128). During surgery, the used anaesthetic (isofluran) probably lowered the blood pressure. During recovery the blood pressure increased again. As a consequence a newly formed blood clot could have failed to seal the incision side and caused bleeding at the anastomosis. Unfortunately, blood pressure was not measured at any time during the study, to confirm this hypothesis. To prevent further bleeding, hypertension could be controlled with medical treatment before and shortly after surgery. An adjustment for the insulin therapy should be considered, because insulin can increases blood pressure shortly after initiation of the treatment. The blood pressure will normalize over time again. Because surgeries were done 1 week after initiation of insulin, the treatment could have contributed to the high number of reactive bleedings in this study (129).

Many rats that had RYGB surgery showed transient anorexia with hypersalivation, till the end of the study. The cause could be a temporary obstruction in the oesophagus or at the gastrojejunal anastomosis, causing the animal to feel sick for a couple of hours. The hypersalivation passed, when animals were given soft food, to facilitate food passage. Metformin can cause GIT stress inducing nausea and diarrhoea (130). Diarrhoea could not be observed in our rats, but hypersalivation, a sign of stress, unease and nausea in rats. Maybe metformin contributed to the clinical signs, however, hypersalivation was also observed in the RYGB rats without medical treatment.

4.4 Post surgery

The main goal of the study was to see if kidney injury could be reduced with different forms of T2DM therapy and which one would be the most successful. Compared were rats having RYGB surgery, 15% weight loss in combination with medical treatment (metformin, ramipril, rosuvastatin and fenofibrate), or a combination of both. The outcomes were compared to the healthy controls (SD) and 9 ZDSD rats that received no treatment.

4.4.1 Diabetes remission

After RYGB surgery the rats lost up to 20 % of their initial body weight. But if they did not receive additional medical treatment post RYGB surgery, they regained weight till the end of the study. The same could be observed for the blood glucose. Hyperglycemia improved after RYGB surgery. But the overall success for diabetes remission was less than in the group that received additional medical treatment. In recent years it became clear, that RYGB surgery is not an absolute cure for T2DM. In a nationwide Swedish cohort study 67% of the patients that underwent RYGB surgery did not need further

medical treatment 2 years post surgery. But the number decreased to 61% seven years post RYGB surgery (131). In another study the initial T2DM remission was 68% within 5 years post RYGB surgery, but 35% relapsed within the next 5 years(132). In that study poor preoperative glycemic control, insulin use and longer T2DM duration were considered risk factors for a relapse. Patients who gained more weight post RYGB surgery had a worse outcome for T2DM remission (132, 133). All this studies in combination with our findings suggest that RYGB surgery mainly reduces the amount of medication prescribed. But for a completely successful T2DM treatment medication can be still needed.

The 15% weight loss and medical treatment group (BMT) had a better diabetes remission rate than RYGB surgery alone. Different studies have shown that weight loss can prolong the onset to diabetes in prediabetic patients, for example the Diabetes Prevention Program (DPP) in the US. In a 15 years follow up the cumulative incidence for the onset of diabetes from prediabetes could be reduced from 62 % in the placebo group to 55% in patients with 7% weight loss and 150 min moderate exercise per week and 56% when treated only with metformin(134). In the Let's Prevent study in the UK took a more pragmatic approach to prevent diabetes. Their goal was to bring the lifestyle intervention into primary care, in a more realistic and cost effective way. They used a risk score in combination with a blood test to determine people at risk. Then they invited the patients to a 6 hour group education session, which were combined with a 3 hour refresher session one and two years after the beginning of the program in combination with a short phone call every three months. The goal was to motivate the people to lose at least 5% of their body weight, reduce fat intake, increase the percentage of monosaturated fats and the fibre intake and encourage moderate exercise (135). In the 3 years follow up a non significant 26% reduction in onset to diabetes could be observed. Smokers and people with lower income were more likely to fail (136). But weight loss is not only beneficial in patients with prediabetes, but also in patients with T2DM. The Action for Health in Diabetes (AHEAD) study found a positive association between 5-10% weight loss with improvement of glycaemia, blood pressure, triglycerides and HDL-cholesterol in T2DM patients in a 1 year follow up. The results were even better when the weight loss was 10-15 % of the initial body weight (137). The ongoing Diabetes Remission Clinical Trial (DiRECT) wants to test T2DM remission in general practice through a special diet that should help the participants to lose > 15 kg of their initial body weight (138). The first results from that study show that 24 % of the participants in the weight loss group could achieve the weight loss goal. T2DM remission was achieved in 86 % of the participant that lost > 15 kg and 46 % when 10-15 kg weight loss. Less anti-diabetic antihypertensive drugs were prescribed in the intervention group (139).

In our study the ZDSD rats with RYGB surgery in combination with medical treatment (RYGB(BMT) had the highest diabetes remission rate. Only one individual was still diabetic 4 weeks post surgery. To control the hyperglycemia after surgery the rats received initially only metformin. Later fenofibrate and ramipril was added. The weight

loosing properties of the RYGB surgery in combination with the weigh reducing effects of the used medication, was probably the main cause of the successful diabetes remission in the RYGB(BMT). Striking was the ongoing weight loss in the RYGB(BMT) group A possible explanation for the ongoing weight loss was the reduced food intake, see Figure 11 Body weight change 4 weeks post surgery and food intake previous study showed that RYGB surgery reduces food intake and enhances weight loss, mainly in the first month post surgery (140). The same could be observed in this study. The main weight loss occurred in the first 10 days post surgery. In addition metformin hydrochloride has been shown to, not only improve hyperglycemia, but also support weight loss (134). One of the main mechanism is a reduction in food intake (141). It can block glucose absorption directly in the intestine (142), it improves leptin and insulin sensitivity (143, 144) and it reduces hepatic gluconeogenesis (145). In addition, different studies have demonstrated that fibrates can inhibit the effect of weight gaining medicaments and even induce mild weight loss on their own (146, 147). For future studies, it would be interesting to know if RYGB in combination with only metformin would be enough to achieve a high diabetes remission rate.

The fact that the sham rats also lost weight, when showing severe T2DM symptoms, demonstrates that mild weight loss is not enough for diabetes remission. Or maybe remission can be achieved only at an early stage of diabetes. This would not be surprising. When T2DM progresses more and more beta cells are destroyed. Insulin production will be permanently reduced. The weight loss is then a consequence of energy waist, because the glucose cannot be used effectively.

4.4.2 Kidney injury

Not only hyperglycemia and obesity improved in the RYGB(BMT) group, but also the albumin excretion rate. The same could be observed in the BMT group. The effect was less prominent in the RYGB group. Sumida et al. (2017) showed that already a minor increase in the urinary albumin excretion rate over one year, has negative effects on the outcome of the kidney disease(116). Reid et al. (2014) showed that albuminuria improved after RYGB surgery and regression from micro- to normoalbuminura was observed in > 80% of the patients (148). Two studies compared RYGB to intensive medical treatment. In both studies the albumin creatinine ratio improved only in the RYGB group (149, 150). However, also most of the RYGB patients received medical treatment in addition to the surgery, so they would correspond to the RYGB(BMT) groups in this study. Indeed, the RYGB(BMT) rats showed an significant improvement of albuminuria 4 weeks post surgery. The improvement was not significant when only RYGB surgery was performed. The results of this study suggest, that the medical supporting treatment is necessary, to improve albuminuria and hyperglycemia.

The BMT group had significant improvements of albuminuria. Ramipril is very effective in reducing albuminuria in patients with T2DM(151). Rosuvastatin can enhance the positive effect of the ACE inhibitors. It reduces the oxidative stress, caused by hyperglycaemia, on the kidney cells. As a consequence cell damage and cell loss is reduced and less protein lost (40).

In a recent study a diabetic rat model (db/db) was treated with fenofibrate. An in improvement of albuminuria and morphological kidney changes was reported (42). Other than the RYGB(BMT) group, the BMT's also received liraglutide, a GLP-1 agonist. Studies have demonstrated the positive effect of Liraglutide when added to the diabetic treatment. Albuminuria could be reduced and progression of CKD in case of DKD was slowed down (152, 153).
A special focus should be put on the weight loss in the BMT group. Afshinnia et al. (2010) showed in a meta analysis, that each 1kg weight loss reduces urinary albumin excretion about 1.1 mg/24h. In conclusion, our results suggest that that RYGB surgery has better outcomes regarding albuminuria when combined with medical treatment. Good medical treatment paired with 15% weight loss, has as good outcomes as RYGB(BMT).

In this study none of the intervention reduced the NGAL excretion into the urine. The RYGB group showed no improvement, but also no increase in the NGAL secretion 4 weeks post surgery. The similar result was published in a study, where 22 severely obese adolescent without microalbuminuria showed that urinary NGAL was not reduced one year after RYGB surgery (114). However these results are not completely comparable, since our rats were diabetic, and adults.

While a significant improvement in albuminuria in the BMT and RYGB(BMT) group was observed, NGAL excretion significantly worsened in the same groups. Some NGAL can be pre-renal in origin as it is small enough to be filtered. Filtered NGAL is normally reclaimed by the same pathway as used for albumin in the proximal tubule. However the correction of albumin, but sustained NGAL excretion, is indicative of ongoing renal injury in the nephron downstream of the proximal tubule. Many of the medicaments used in this study can have negative effects on the kidney. Fenofibrate or rosuvastation and ACE inhibitors can cause acute kidney injury, if overdosed(154), resulting in an elevation of NGAL (155, 156). In this study none of the rats received an individual medical treatment plan. A slight overdosing in some of the animals could be possible. However most likely the elevated urinary NGAL were not high enough to speak of an AKI in the RYGB(BMT) or BMT animals. To evaluate which drug is responsible for these changes, further studies have to be made. Urinary NGAL concentrations have been associated with progression of chronic kidney disease (81). Compared to KIM-1, NGAL levels in the urine can be associated with the severity of albuminuria (157).

Urinary KIM-1 excretion rate was low in all groups before the intervention. However, its urinary excretion rate increased, when the animals did not receive any treatment, but was not significant. It did not change in the BMT, RYGB and RYGB(BMT) group. Like NGAL, KIM-1 is a marker for proximal tubulus damage. High urinary KIM-1 excretion has been associated with a faster progression of DKD, because of a faster decrease of the kidney function (GFR) (82). Increased urinary KIM-1 and NGAL excretion in combination with microalbuminuria enhances the risk for ESRD and death further (158). Carvahlo et al. (2016) showed that an increase of urinary NGAL or KIM-1 can

occur before the onset of microalbuminuria (159). This was not the case in our study. But we used a rat model instead of humans.

Most studies focus on serum OPN rather than urinary OPN, after Yamagucci et al. (2004) showed that only plasma OPN correlated with the progression of DKD to ESRD. The study involved 229 patients with T2DM (121). Another preliminary study showed that a lower serum OPN is associated with increased insulin sensitivity, and more body weight loss after RYGB surgery and a overall improvement of the outcome (160). However, in our study we found a positive correlation between albuminuria and urinary OPN excretion rate and a correlation between urinary OPN and the degree of hyperglycaemia. Interestingly, the interventions like RYGB surgery or medical treatment did not decrease the OPN excretion rate. Instead a increase in the urinary OPN was observed in the BMT RYGB and SD group. Plus there was a non significant trend for elevated urinary OPN in the sham group. The elevation in the SD and sham group could be explained with an increase in bodyweight an obesity (11). The increase in the BMT group was surprising. A reduction of urinary OPN was expected, because ramipril has been shown to reduce the gene expression of OPN in the kidney. However, if ACE inhibitors can prevent tubulointerstitial fibrosis stays controversial (161, 162).

In summary, not all animal could be treated successfully with RYGB surgery alone If medical treatment was added to the surgery, the best outcomes could be observed. Interestingly 15% weight loss in combination with oral medical treatment was almost as successful in improving hyperglycaemia as the RYGB(BMT) combination. The most surprising finding was, that with the improvement of albuminuria in the RYGB(BMT) and BMT group NGAL excretion rate increased. Overdosing of the medication, could be a possible explanation for these findings. To prove this, further experiments with individual treatment plans or different drug combinations are needed. Further, in this study the term kidney damage is used, referring to elevated kidney injury markers. To evaluate the effective kidney damage, histology will be needed.

4.4.3 Limitations

Tight management of weight loss was challenging and may have influenced the results.

As medication was given with the food, precise dosing may not have occurred

The medication was not adjusted for every individual, as it would be in humans. Some rats might have received medical treatment that was not necessary.
In general, this study should be completed with the histological findings of the kidneys.

4.4.4 Conclusion

Only about 50% of the ZDSD rats were diabetic at the age of 26 weeks. This was a lower number than expected. ZDSD rats within the lowest blood glucose quartile had less kidney injury and showed no diabetic symptoms compared to the rats within the upper glucose quartile, when 26 weeks old. A significant difference could be observed

regarding, polyuria, polydipsia, polyphagia and the amount of excreted kidney injury markers into the urine between the rats from the upper and lower glucose quartile.

Different to other studies, we found a correlation between hyperglycaemia and the urinary excretion rate of all testes kidney injury markers (albumin, NGAL, OPN, KIM-1) at the age of 26 weeks. There was no correlation present when the animals were younger, and no correlation was assessed at a later time point. The most interesting finding was the correlation of hyperglycaemia with urinary OPN.

The ZDSD rats that underwent RYGB surgery did not show any significant improvement regarding hyperglycaemia or the concentration of kidney injury markers in the urine. Combination of surgery with medical treatment had better outcome. Hyperglycaemia was controlled better and albuminuria reduced more effectively. No change in the KIM-1 excretion could be observed. Interestingly, an elevated NGAL excretion could be observed in the RYGB(BMT) and in the BMT group 4 weeks after start of the intervention, surprising when contrasted with effect on urinary albumin excretion and suggestive of ongoing kidney injury.

In conclusion this study has deepened insights into the diabetic phenotype in ZDSD rats and the associated degree of DKD. The relative impact of intensive interventions of weight, metabolic control and urinary surrogates of renal injury has provided some thought-provoking data suggesting that remission of albuminuria may not be entirely indicative of reduced renal injury.

5 Literature

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6 Appendix

6.1 Insulin optimisation protocol

The Glucose target from the morning glucose was 6 - 9mmol/L. Over seven days, glucose was measured daily in the morning and then insulin degludec (Tresiba®, Novo Nordisk A/S) was injected sc.
The starting dose of insulin was defined as following:

Table 3 Insulin starting dose

Insulin starting dose	
Blood glucose level in the morning	**Insulin (Units)**
< 9	0
9 - 13	4
13 -17	6
17 - 21	8
21 - 25	10
> 25	12

The insulin dose was adjusted every two to three days following the protocol in Table 4., Because many ZDSD responded to a new insulin dose after 3 days and not two. The maximum dose of 20 units per rat was not exceeded, to reduce the risk of hypoglycaemia.

Table 4Insulin adjustments

Insulin dose adjustments	
Blood glucose level in the morning	**Insulin (Units)**
< 6	-4
6 – 9	0
9 – 13	+4
13 – 17	+6
17 – 21	+8
21 - 25	+10
> 25	+12

The day before surgery only half the units from the day before were given.

6.2 Liraglutide Titration Protocol

Liraglutide was started at the age of 30 weeks in the BMT group. The dose was adjusted every day according to Table 5. Water consumption was monitored, to prevent hypodipsia and dehydration. The ZDSD rats became anorexic, when administrated 0.5mg/kg of Liraglutide. The dose was kept on 0.5mg/kg for another day then the treatment was stopped, because of the animal welfare. Liraglutide was reintroduced when the ZDSD rats were back to 10% weight loss on a constant rate of 0.2 mg/kg, till the end of the study.

Table 5 Liraglutide titration adjustments

Liraglutide Titration	
Day	**Liraglutide [mg/kg]**
1	0.025
2	0.025
3	0.05
4	0.1
5	0.15
6	0.2
7	0.3
8	0.4
9	0.5

6.3 Rat scoring system

For pain evaluation of the animals during the study the rat grimace scale was used, that was developed by Sotocinal et al. (2011) (163). If the pain score was more than 3, a 0.05mg/kg Buprenorphin subcutaneous injection was given. In addition skin lesions, wounds and discharge were reported. To evaluate further actions and possible endpoints during the study, Table 6 was used.

Table 6

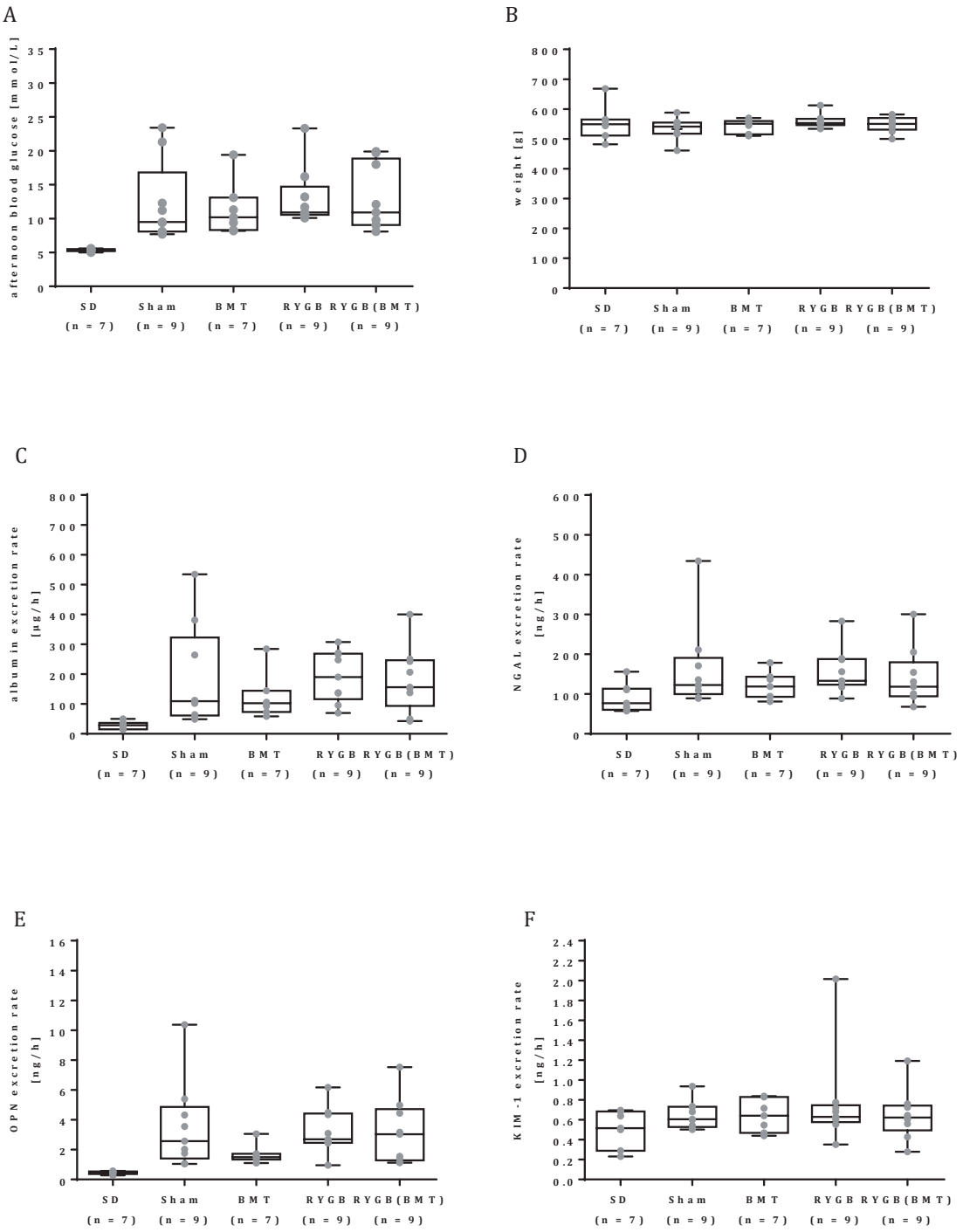
Feature	Score	Description
Appearance	0	Normal
	1	General lack of grooming/mild dehydration
	2	Dehydration/hunched
	3	Severe dehydration
Weight	0	weight loss less than 5%
	1	Body weight < 10% decrease
	2	Bodyweight < 10-20% decrease
	3	Bodyweight < 30% decrease
Behaviour	0	Normal
	1	Minor depression or exaggerated response
	2	Decreased mobility or alertness/isolation
	3	Vocalisation/restless or still/precomatose
Respiratory	0	normal
	1	slight changes/increased rate only
	2	Increased rate with abdominal breathing
	3	Decreased rate with abdominal breathing
Actions:	Score 0	Normal – no action
	Score 1-4	Observation, consider action
	Score 5-7	Suffering administration analgesia/fluids
		Euthanasie if a) Score of 3 in any single category, except weigth* b) total score of 7 or above, c) other indications

* A 30% weight loss in the rats with RYGB surgery is expected. If the rat only scored 3 in weight, but looks healthy, the necessity of euthanasia can be discussed

6.4 Parameters of the study groups before intervention

The afternoon blood glucose was the same in Sham 9.5 mmol/L (8.1 – 12.3), BMT 9.8 mmol/L (8.2 – 12.7), RYGB 10.9 mmol/L (10.4 – 14.0) and RYGB(BMT) 10.9 mmol/L (9.1 – 18.9). The median blood glucose of the SD was 5.3 mmol/L (5.2 – 5.5). The weight was the same in Sham 520g (517 – 556), BMT 548g (517 – 559), RYGB 558g (549- 567) and RYGB(BMT) 550g (531 – 570), Kruskal-Wallis two tailed. The weight of the SD was 549g (511 – 565). There was no difference in albumin loss into the urine for sham 109 µg/h (60 - 323), BMT 103 µg/h (73 – 144), RYGB 190 µg/h (116 – 268) RYGB(BMT) 156 µg/h (93 - 247), Kruskal-Wallis, two tailed. The AER of the SD was 28 µg/h (28 - 13). There was no difference in the NGAL concentration in the urine for sham 122 ng/h (100 - 191), BMT 119 ng/h (93- 143), RYGB 133 ng/h (124 - 188), RYGB(BMT) 118 ng/h (94 - 180), Kruskal-Wallis two tailed. The NGAL excretion of the of the SD was 77 ng/h (60 - 133). There was no difference in the OPN concentration in the urine for sham 2.57 ng/h (1.41 – 4.86), BMT 1.50 ng/h (1.34 – 1.72), RYGB 2.70 ng/h (2.45 – 4.42), RYGB(BMT) 3.03 ng/h (1.28 – 4.71), Kruskal-Wallis two tailed. The OPN excretion rate of the SD was 0.48 ng/h (0.37 – 0.53).

There was no difference in the KIM-1 concentration in the urine for sham 0.605 ng/h (0.528 – 0.730), BMT 0.641 ng/h (0.468 – 0.829), RYGB 0.630 ng/h (0.577 – 0.746), RYGB(BMT) 0.622 ng/h (0.494 – 0.743), Kruskal-Wallis two tailed. The KIM-1 excretion rate of the SD was 0.515 ng/h (0.289 – 0.683).

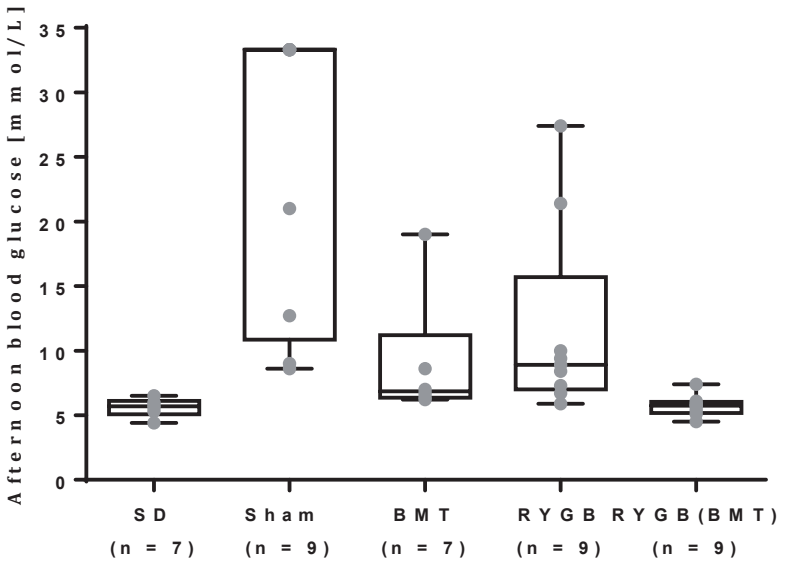


Supplementary 1 Parameters of the different groups before intervention

Graph A-F show the median and 25th – 75th quartile, inclusive minimum and maximum values of the afternoon blood glucose, weight, albumin, NGAL, OPN, and Kim-1 excretion rate of each study group at 26 weeks. each dot represents an individual.

6.5 Glucose values of the intervention groups four weeks post intervention

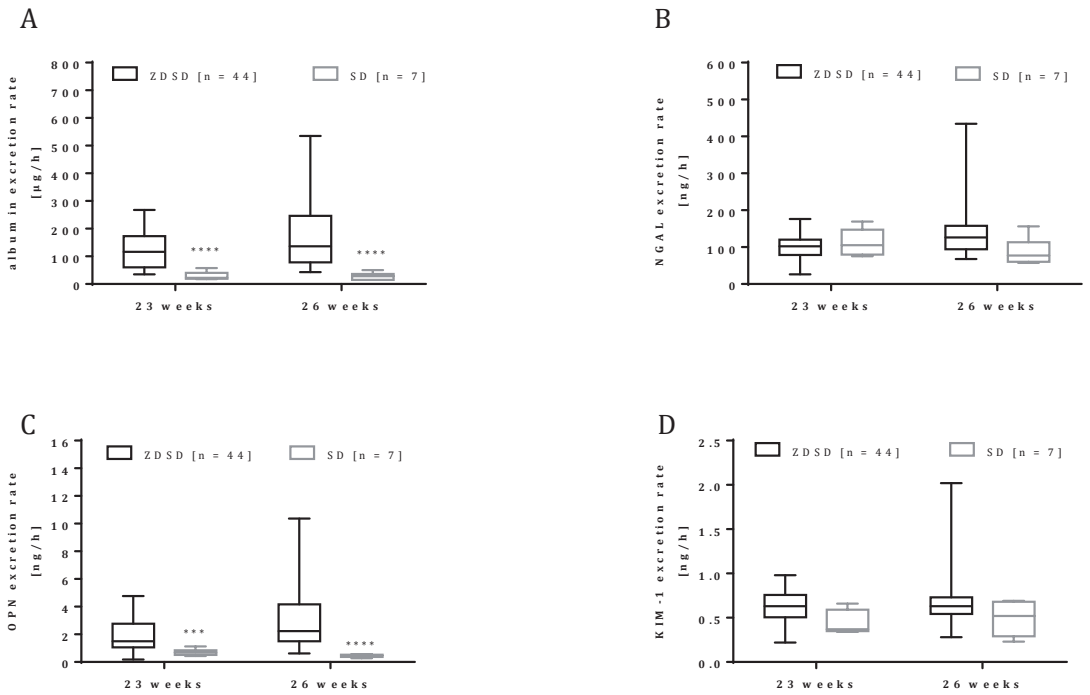
The median of the blood glucose did not differ between the sham, BMT RYGB and RYGB (BMT) 4 weeks post surgery 33.3 mmol/L (10.9 – 33.3), 6.9 mmol/L (6.3 – 11.2), 8.9 mmol/L (7.0 – 15.7), 5.8 mmol/L (5.2 – 6.1), Kruskal-wallis, two tailed. The medium of the SD was 5.7 mmol/L (5.1- 6.2).



Supplementary 2 Afternoon blood glucose four weeks post intervention
The graph shows the median and 25th – 75th quartile, inclusive minimum and maximum values of the afternoon blood glucose. Each dot represents an individuum.

6.6 Comparison of the urinary kidney injury markers of ZDSD and SD rats at the age of 23 and 26 weeks

When 23 weeks old the ZDSD rats lost significantly more albumin into the urine compared to the SD rats (control) with a median of 116 µg/h (60 -173) compared to 22 µg/h (19 – 40), p < 0.0001, Mann Whitney U, two tailed. This did not change when they were 26 weeks old. The ZDSD had an albumin excretion rate of 137 µg/h (78 – 246) compared to 28 µg/h (15 – 36), p < 0.0001 , Mann-Whitney U, two tailed. The NGAL excretion rate into the urine was 102 ng/h (78 – 120) for ZDSD and 105 ng/h (79 – 147) for the control, at 23 weeks and 126 ng/h (94 – 158) respectively 77 ng/h (60 – 113) at 26 weeks. At both time points there was no difference between the SD and the ZDSD rats. The 23 weeks old ZDSD rats showed already an elevated OPN excretion with 1.51 ng/h (1.06 – 2.77) compared to the control (SD rats) 0.68 ng/h (0.51 – 0.85), p = 0.0007, Mann-Whitney U, two tailed. The same was observed when 26 weeks old. ZDSD excreted 2.24 ng/h (1.50 – 4.17) and the SD 0.48 ng/h (0.37 – 0.53), p <0.0001, Mann-Whitney U, two tailed. The KIM-1 excretion rate into the urine was 0.630 ng/h (0.505 – 0.758) for ZDSD and 0.370 ng/h (0.350 – 0.590) the control, at 23 weeks and 0.630 ng/h (0.543 – 0.730) respectively 0.520 ng/h (0.290 – 0.680) at 26 weeks. At both time points there was no difference between the SD and the ZDSD rats.



Supplementary 3 Kidney injury markers excretion into urine at the age of 23 and 26 weeks

The graphs show the median and 25th – 75th quartile, inclusive minimum and maximum values of the albumin excreation rate (A), NGAL (B), OPN (C) and KIM-1 excretion rate. *** = p-value < 0.001, **** = p-value < 0.0001, Mann-Whitney U test, two tailed.

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Curriculum Vitae

Vorname Name	Michaela Hutter
Geburtsdatum	06.01.1993
Geburtsort	Altstätten SG
Nationalität	Schweizerin
Heimatort	Oberriet-Kriessern SG

08/1999 – 07/2005	Primarschule, Kriessern, Schweiz
08/2005 – 07/2007	Sekundarschule, Montlingen, Schweiz
08/2007 – 07/2011	Kantonsschule Heerbrugg, Heerbrugg Schweiz
29.06.2011	Matura, Kantonsschule Heerbrugg, Heerbrugg, Schweiz
09/2012 – 08/2017	Studium der Veterinärmedizin, Vetsuisse Fakultät, Universität Zürich, Schweiz
01/2018	Abschlussprüfung vet. med. Universität Zürich, Schweiz
03/2018 – 03/2019	Anfertigung der Dissertation unter Leitung von Prof. Dr. med. vet. Thomas A. Lutz am Institut für Veterinärphysiologie der Vetsuisse-Fakultät Universität Zürich Direktor: Prof. Dr. med. vet. Max Gassmann
08/2018 – bis jetzt	Intern in der Pferdeklinik, University of Dublin Veterinary Hospital, Dublin, Irland